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13. ABSTRACT *(Maximum 200 Words)*

To gain a better understanding of how estrogen-responsive genes are regulated we utilized *in vivo* footprinting and *in vitro* binding assays to define cis elements and trans acting factors involved in conferring estrogen responsiveness to the pS2 and PR genes in MCF-7 breast cancer cells. The consensus pS2 ERE half site was protected in the absence of hormone and both the consensus and imperfect ERE half sites were protected in the presence of estrogen. 4-hydroxytamoxifen and ICI 182,780 elicited distinct footprinting patterns, which differed from those observed with vehicle- or with estrogen-treated cells. Footprinting patterns in and around the TATA and CAAT sequences were identical in the presence and in the absence of estrogen suggesting that the basal promoter is accessible and poised for transcription even in the absence of hormone. *In vitro* DNase I footprinting experiments demonstrated that the estrogen receptor bound to the pS2 ERE and that adjacent nucleotides were protected by MCF-7 nuclear proteins. The PR gene is also induced by E_2 in MCF-7 cells. Although the PR gene lacks an identifiable ERE, it does contain a complex array of conserved transcription factor binding sites. We have provided evidence that two AP-1 sites help to confer estrogen responsiveness to the PR gene. In addition the ER enhances Sp1 binding to its recognition site and binds directly to an adjacent ERE half site in the PR Promoter A. Taken together, our combined studies of the pS2 and the PR gene suggest that the ER has direct and indirect effects on formation of an active transcription complex.

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Table of Contents

INTRODUCTION

My overall goal has been to better understand how the estrogen-responsive genes are regulated. During the tenure of my support from the DOD, I have studied the estrogen-responsive pS2 and progesterone receptor (PR) genes using *in vivo* and *in vitro* approaches. Exposure of MCF-7 human breast cancer cells to 17β-estradiol (E_2) activates transcription of the single-copy pS2 gene (1) and results in increased levels of pS2 mRNA and secreted protein. Transient transfection assays have demonstrated that a single imperfect estrogen response element (ERE) confers estrogen-responsiveness to this gene (2). The PR gene is also under estrogen control in MCF-7 cells (3-5). PR mRNA and protein levels increase and are maximal after three days of E_2 treatment of MCF-7 cells (3-5). Unlike the pS2 gene, no consensus EREs have been identified in the PR gene (6). I have used *in vivo* ligation mediated polymerase chain reaction (LMPCR) footprinting analysis, transient transfections, and *in vitro* binding assays to define cis elements and trans acting factors involved in conferring estrogen responsiveness to the pS2 and PR genes in MCF-7 cells. The results of my studies are included in two published manuscripts and one manuscript, which has been submitted and is being revised.

BODY
Statement of Work:

Task 1: Determination of experimental conditions resulting in clear footprint ladders using the LMPCR protocol.

Task 2: Optimization of DNase I treatment conditions and LMPCR conditions for DNase I treated genomic DNA.

Task 3: Detection of *in vivo* footprinting of the TATA and CAAT boxes of genomic DNA from MCF-7 cells treated with ethanol of 17 β-estradiol.

Task 4: Culture of MCF-7 cells and treatment of cells with antiestrogens, trans-hydroxytamoxifen or ICI 182,780. DNase I treatment of cells. Detection of *in vitro* footprinting of the TATA and CAAT boxes of genomic DNA from MCF-7 cells treated with trans-hydroxytamoxifen or ICI 182,780.

Task 5: Optimization of conditions for primers designed to examine the pS2 ERE.

Task 6: Culture of MCF-7 cells and treatment of cells with ethanol, 17 β-estradiol, trans-hydroxytamoxifen or ICI 182,780. DNase I treatment of cells.

Task 7: Detection of *in vivo* footprints of the pS2 ERE.

Completion of experiments outlined in the Statement of Work
Task 1-2: DNase I treatment and LMPCR conditions that result in clear footprinting ladders have been optimized to examine the pS2 gene.
Task 3: MCF-7 cells were treated with ethanol or 17β-estradiol, DNase I treated and genomic DNA isolated. *In vivo* footprints were detected on the noncoding strand in the regions of the TATA and CAAT boxes. The TATA and CAAT sequences were protected and flanked by hypersensitive sites in the presence and in the absence of hormone in estrogen receptor (ER)-positive MCF-7 cells but not ER-negative MDA MB 231 cells (Ref. 7, Appendix #1). These findings indicate that the ER is involved not only in mediating the cellular response to hormone, but may also be involved in organization of the proximal promoter in the absence of hormone. In addition to these objectives stated in our original grant, we extended our investigations to examine dimethlysulfate *in vivo* footprints on the noncoding strand in the regions of the TATA and CAAT boxes.
Task 4: MCF-7 cells were treated with trans-hydroxytamoxifen or ICI 182,780 and DNase I treated, but the regions of the TATA and CAAT boxes were not examined.
Task 5-7: MCF-7 cells were treated with ethanol, 17β-estradiol, trans-hydroxytamoxifen or ICI 182,780 and DNase I treated. *In vivo* footprints were detected with one set of nested primers designed to examine the noncoding strand of the pS2 ERE. In addition to these objectives stated in our original grant, we extended our investigations to examine dimethlysulfate *in vivo* footprints on the noncoding strand in the region of the pS2 ERE. Examining the regulatory elements of the pS2 gene utilizing *in vivo* DNase I footprinting demonstrated that 24 hour

estrogen treatment of MCF-7 cells resulted in extensive protection within and adjacent to the pS2 ERE. Interestingly treatment of MCF-7 cells with the antiestrogens 4-hydroxytamoxifen or ICI 182,780 each produced distinct protection patterns of the pS2 ERE.

Additional experiments with the pS2 gene

        A number of experiments were completed to more fully understand how the pS2 gene is regulated by estrogen. These experiments were not outlined in the original Statement of Work. These studies have been reported in work published by Kim et al (7). This manuscript has been included as Appendix #1.

        We compared the time frame of pS2 mRNA expression to events occurring at the level of the gene. Northern blot analysis was utilized to detect the relative levels of pS2 mRNA. The level of MCF-7 pS2 mRNA increased 16-fold after a 24 hours exposure to estrogen. These findings demonstrated that the pS2 gene was essentially inactive in the absence of estrogen, but responded robustly to estrogen treatment.

        We further extended our studies to identify proteins interacting with the pS2 ERE. To determine whether proteins present in MCF-7 nuclear extracts could bind to the pS2 ERE, gel mobility shift assays were carried out. When DNA fragments containing the pS2 ERE were combined with purified ER or nuclear extracts prepared from estrogen-treated MCF-7 cells, a major protein-DNA complex was formed. Since we anticipated that ER might bind to this region, antibodies to this proteins was included in separate binding reactions. The major protein-DNA complex was supershifted by the ER-specific antibody, H151, suggesting that ER bound efficiently to the pS2 ERE.

        To aid in the identification of proteins associated with the ERE bound ER, *in vitro* DNase I footprinting experiments were utilized, and the resulting protection patterns compared to our *in vivo* footprinting experiments. DNA fragments, each containing the pS2 ERE, or the pS2 TATA box and CAAT box were incubated with increasing amounts of estrogen-treated MCF-7 nuclear extract or increasing amounts purified ER and exposed to DNase I. The resulting protection patterns were very similar to the protection patterns of our *in vivo* footprinting experiments. The pS2 perfect ERE half site was protected at the lowest protein concentrations followed by protection of the imperfect ERE half site at the highest protein concentrations. The CAAT box showed minimal protection with hypersensitive DNase I cleavage sites surrounding both the TATA and CAAT boxes. Because of similar protection patterns in both our *in vitro* and *in vivo* footprinting experiments, it seems likely that proteins present in nuclear extracts from estrogen-treated MCF-7 cells bind to the pS2 regulatory elements in a fashion that is very similar to binding of proteins in intact MCF-7 cells. Taken together our results have helped identify proteins involved in the transcriptional regulation of the pS2 gene.

Progesterone Receptor Gene Regulation

        The goal in the Dr. Nardulli's laboratory is to gain a better understanding of how estrogen-responsive genes are regulated. Dr. Nardulli was awarded one Idea Award to examine pS2 gene regulation and another Idea Award to study PR gene regulation. Therefore, I have extended my investigation of estrogen-responsive genes to studying the human progesterone receptor (PR) gene. The PR gene was particularly interesting because it is an estrogen-responsive gene, but lacks an identifiable ERE. My work on the PR gene has been described in two manuscripts. One by Petz et al describes the involvement of the half ERE/Sp1 binding site

in PR gene expression is summarized below. A reprint of this manuscript is included as Appendix #2.

Two distinct PR forms are differentially expressed in a tissue-specific manner (8-12). PR-B is a 120 kD protein containing a 164 amino acid amino-terminal region that is not present in the 94 kD PR-A. Two discrete promoters, A and B, which are thought to be responsible for the production of PR-A and PR-B, respectively, have also been defined (6). The activities of these two promoters are increased by estrogen treatment of transiently transfected Hela cells. Interestingly, no consensus EREs have been identified in either Promoter A (+464 to 1105) or Promoter B (-711 to +31). Promoter A does, however, contain an ERE half site located upstream of two Sp1 sites (6). The presence of these adjacent binding sites suggests that the ER might be able to influence PR expression directly by binding to the ERE half site, indirectly by interacting with proteins bound to the putative Sp1 sites, or a combination of these two methods. We have used *in vivo* DNase I footprinting to demonstrate that the half ERE/Sp1 binding site is more protected when MCF-7 cells are treated with estrogen than when cells are not exposed to hormone suggesting that the this region is involved in estrogen-regulated gene expression. When cells were treated with E_2 for 2 hours, the protection of the proximal Sp1 site, the distal Sp1 binding site, and the ERE half site was greater than seen in cells that had not been exposed to hormone (Ref. 13, Appendix #2). After 72 hours of E_2 treatment, a time when PR mRNA and protein reach maximal levels (3-5, 14, 15), the protection of the half ERE/Sp1 binding site was sustained. E_2 treatment also elicited protection of regions flanking the half ERE/Sp1 binding site. The ability of the half ERE/Sp1 binding site to confer estrogen responsiveness to a simple heterologous promoter was confirmed in transient cotransfection assays. Exposure of transiently cotransfected CHO cells to E_2 resulted in increased CAT activity when the reporter plasmid contained the half ERE/Sp1 binding site compared to the parental reporter plasmid. *In vitro* DNase I footprinting and gel mobility shift assays demonstrated that Sp1 present in MCF-7 nuclear extracts and purified Sp1 protein bound to the two Sp1 sites, first to the proximal then to the distal Sp1 site, and that the estrogen receptor enhanced Sp1 binding. In addition to its effects on Sp1 binding, the estrogen receptor also bound directly to the ERE half site.

The final portion of my studies delineated the role of two AP-1 sites in regulating PR gene expression. This work by Petz et al has been submitted to Molecular Endocrinology and is in revision at the present time. This manuscript is included as Appendix #3. In this work I identified two putative activating protein-1 (AP-1) sites at +90 and +745 in the PR gene and tested the sites for their abilities to bind to Fos and Jun and function as transcriptional enhancers. Genomic Southern analysis was utilized to identify a region at approximately +90 of the PR gene that was hypersensitive to DNase I cleavage. The susceptibility of this region to DNase I cleavage is most likely due to loose association of the DNA with histones in this region. Analysis of nucleotide sequence at the +90 hypersensitive site identified a putative AP-1 site (TGAGT<u>GA</u>) from +90 to +96 that differs from a consensus AP-1 site (TGAG/CTCA, Ref. (16, 17) by one nucleotide. I also identified a putative AP-1 site (TGACT<u>GA</u>) in the region from +745 to +751 of the PR gene, that differs from a consensus AP-1 site (16, 17) by one basepair. The location and nucleotide sequence of the +745 AP-1 site are completely conserved in mouse (18), rat (19), rabbit (20), and human (6) PR genes. The +90 and +745 AP-1 sites bound purified Fos and Jun, formed complexes with Fos-Jun heterodimers present in MCF-7 nuclear extracts, and functioned as estrogen-responsive enhancers in transient cotransfection assays. Estrogen treatment of MCF-7 cells transiently increased Fos levels and elicited a decrease in phosphorylated Jun and a concomitant increase in the dephosphorylated Jun. Chromatin

immunoprecipitation assays demonstrated that the estrogen receptor was present at the +90 and +745 AP-1 sites in the endogenous PR gene after treatment of MCF-7 cells with estrogen. Estrogen treatment of MCF-7 cells transiently increased Fos levels and elicited a decrease in phosphorylated Jun and a concomitant increase in the dephosphorylated Jun. These studies suggest that the ER cooperates with Fos and Jun at the +90 and +745 AP-1 sites to assist in conferring estrogen responsiveness to the PR gene. Taken together, these findings suggest that multiple cis elements and trans acting factors are involved in conferring estrogen responsiveness to the PR gene.

KEY RESEARCH ACCOMPLISHMENTS:

Involvement of an imperfect ERE in estrogen-induced pS2 gene expression
- *In vivo* DNase I footprinting experiments indicate that:
    * events occurring at the level of the pS2 gene are well coupled to the levels of pS2 mRNA in estrogen-treated MCF-7 human breast cancer cells.
    * the pS2 ERE is strongly protected after estrogen treatment and remains occupied for at least 24 hours after treatment.
    * antiestrogens mediate their effects by promoting the association of proteins with unique regions of the pS2 promoter.
    * the pS2 TATA and CAAT sequences are occupied and are flanked by hypersensitive sites in the presence and in the absence of estrogen-treatment.
- Purified ER and ER present in estrogen-treated MCF-7 cells can bind to the pS2 ERE, and is supershifted by an ER-specific antibody.
- *In vitro* DNase I footprinting experiments result in protection patterns that are very similar to the protection patterns seen in *in vivo* footprinting experiments of the pS2 gene.

Involvement of the half ERE/Sp1 binding site in estrogen-induced PR gene expression
- *In vivo* DNase I footprinting experiments indicate that the half ERE/Sp1 binding site is protected after estrogen treatment of MCF-7 cells.
- Estrogen enhances transcription of a reporter plasmid containing the half ERE/Sp1 binding site.
- ER enhances binding of Sp1 to first the proximal then the distil Sp1 binding site.
- ER binds directly to the half ERE site.
- Sp1 and ER form a stable trimeric complex with the half ERE/Sp1 binding site.

Involvement of AP-1 sites in estrogen-induced PR gene expression
- The +90 Ap1 site is hypersensitive to DNase I cleavage in MCF-7 cells.
- Estrogen enhances transcription of a reporter plasmid containing the +90 and the +745 Ap1 binding site.
- The +90 and +745 AP-1 sites bound purified Fos and Jun and formed complexes with Fos-Jun heterodimers present in MCF-7 nuclear extracts
- Fos levels increased and Jun is dephosphorylated after estrogen treatment of MCF-7 cells.
- ER is present at the +90 and +745 Ap1 sites in native chromatin.

REPORTABLE OUTCOMES

Publications

Petz, L.N., Ziegler, Y.S., Loven, M.A. and Nardulli, A.M. (2001) AP-1 Sites Are Involved in Conferring Estrogen Responsiveness to the Human Progesterone Receptor Gene. *Molec. Endocrino.* (in revision).

Kim, J.S., Petz, L.N., Ziegler,Y., Wood J.R., Potthoff, S.J., and Nardulli, A.M. (2000) Mechanisms Controlling pS2 Gene Expression: Effects of Estrogen and Antiestrogens on Basal Promoter Elements. *J. Steroid Biochem. Molec. Biol.* 74:157-168.

Petz, L.N., and Nardulli, A.M. (2000) Regulation of the Progesterone Receptor Gene by Estrogen in MCF-7 Human Breast Cancer Cells. *Molec. Endocrino.* 14:972-985.

Abstracts

Petz, L.N., Ziegler, Y.S., Loven, M.A. and Nardulli, A.M. (2001) AP-1 Sites Are Involved in Conferring Estrogen Responsiveness to the Human Progesterone Receptor Gene. The 83th Endocrine Society, Denver, Colorado. P3-428:540 (abstract).

Petz, L.N., Nardulli, A.M. (1999) Regulation of the Progesterone Receptor Gene by Estrogen in MCF-7 Human Breast Cancer Cells. The 81th Endocrine Society, San Diego, California. P1270:192 (abstract).

Nardulli, A.M., Kim, JS., Potthoff, S.J., Petz, L.N. (1997) *In vivo* Footprinting of the pS2 Gene in Human Breast Cancer Cells. The Department of Defense Breast Cancer Research Program, Washington, DC. 1:171-172. (abstract).

Potthoff, S.J., Petz, L.N., Nardulli, A.M. (1997) Expression of the Estrogen-Regulated pS2 Gene in MCF-7 Human Breast Cancer Cells. The 79th Endocrine Society, Minneapolis, Minnesota. P3499:561. (abstract).

Seminars

Reproductive Biology Training Grant seminar series	1998,1999,2000 and 2001
Allerton Reproductive Biology seminar	1998 and 2001
Twenty-first Annual Minisymposium on Reproductive Biology	2000

Grants submitted using data from DOD projects

Illinois Department of Public Health Fellowship (for L. Petz)	January 2001
NIEHS Training program in Toxicology (for L. Petz)	June 2001
New NIH R01 Application	June 2001

CONCLUSIONS

To understand how hormones and antihormones regulate transcription of estrogen-responsive genes, *in vivo* footprinting was used to examine the endogenous pS2 gene in MCF-7 human breast cancer cells. While the consensus pS2 ERE half site was protected in the absence of hormone, both the consensus and imperfect ERE half sites were protected in the presence of estrogen. 4-hydroxytamoxifen and ICI 182,780 elicited distinct footprinting patterns, which differed from those observed with vehicle- or with estrogen-treated cells suggesting that the partial agonist/antagonist and antagonist properties of 4-hydroxytamoxifen or ICI 182,780, respectively, may be partially explained by modulation of protein-DNA interactions. Footprinting patterns in and around the TATA and CAAT sequences were identical in the presence and in the absence of estrogen suggesting that the basal promoter is accessible and poised for transcription even in the absence of hormone. *In vitro* DNase I footprinting experiments demonstrated that the estrogen receptor bound to the pS2 ERE and that adjacent nucleotides were protected by MCF-7 nuclear proteins. These findings indicate that transcription of the pS2 gene is modulated by alterations in protein binding to multiple sites upstream of the basal promoter, but not by changes in protein-DNA interactions in the basal promoter.

The PR gene is also induced by E_2 in MCF-7 cells. Although Promoters A and B impart E_2 responsiveness to the PR gene, neither promoter contains an identifiable ERE. The 5' flanking region, however, does contain several putative transcription factor binding sites. The sequence and location of an ERE half site, a CAAT sequence, and Sp1 and AP-1 binding sites are highly conserved in these PR genes. This high degree of conservation suggests that multiple regions in the 5' flanking region are important in modulating PR gene expression. Thus, estrogen responsiveness of the PR gene may be imparted by the cooperative action of numerous regulatory sites rather than a single ERE. I have identified three regulatory sites involved in the estrogen-regulation of the PR gene. Two AP-1 sites help to confer estrogen responsiveness to the PR gene through the AP-1 binding proteins Fos and Jun. In addition the ER enhances Sp1 binding to its recognition site and binds directly to an adjacent ERE half site in the PR Promoter A. Taken together, our combined studies suggest that the ER has direct and indirect effects on formation of an active transcription complex and that multiple transcription factors including Fos, Jun, ER, and Sp1 act in concert to confer estrogen responsiveness to the PR gene.

These combined studies have helped to elucidate mechanisms by which estrogen alters transcription of two estrogen-responsive genes. The pS2 gene is an example of a classical ERE-containing estrogen-responsive gene. The PR gene represents a particularly intriguing model to study a nontraditional estrogen-responsive gene, which lacks an identifiable ERE. We have defined mechanisms by which these classical and nontraditional genes are regulated by hormone and delineated cis elements and trans acting factors that bring about these effects. We believe that other sites are also involved in estrogen-regulated expression of the PR gene. Identifying additional cis elements responsible for estrogen-regulated PR gene expression and the proteins that bind to these recognition sequences will be the focus of my future studies.

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APPENDICES

1.) Kim, et al. (2000) *J. Steroid Biochem. Molec. Biol.* 74:157-168.
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Regulation of the estrogen-responsive pS2 gene in MCF-7 human breast cancer cells

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Abstract

To understand how hormones and antihormones regulate transcription of estrogen-responsive genes, in vivo footprinting was used to examine the endogenous pS2 gene in MCF-7 cells. While the consensus pS2 estrogen response element (ERE) half site was protected in the absence of hormone, both the consensus and imperfect ERE half sites were protected in the presence of estrogen. 4-Hydroxytamoxifen and ICI 182,780 elicited distinct footprinting patterns, which differed from those observed with vehicle- or with estrogen-treated cells suggesting that the partial agonist/antagonist and antagonist properties of 4-hydroxytamoxifen or ICI 182,780, respectively, may be partially explained by modulation of protein-DNA interactions. Footprinting patterns in and around the TATA and CAAT sequences were identical in the presence and in the absence of estrogen suggesting that the basal promoter is accessible and poised for transcription even in the absence of hormone. In vitro DNase I footprinting experiments demonstrated that the estrogen receptor bound to the pS2 ERE and that adjacent nucleotides were protected by MCF-7 nuclear proteins. These findings indicate that transcription of the pS2 gene is modulated by alterations in protein binding to multiple sites upstream of the basal promoter, but not by changes in protein-DNA interactions in the basal promoter. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Estrogen; Antiestrogen; Transcription; Chromatin

1. Introduction

Estrogen is a hormone of crucial importance in the development and maintenance of normal reproductive function and is also involved in initiation and proliferation of mammary tumors. Estrogen's actions are mediated through the intracellular estrogen receptor (ER), which interacts with estrogen response elements (EREs) in target genes to initiate changes in gene transcription [1]. In addition to the previously identified ERα, a second receptor, ERβ, has been identified and has demonstrated the ability to induce transcription of ERE-containing reporter plasmids [2,3].

The pS2 gene is an estrogen-responsive gene, which is expressed in breast cancer cells, but not in normal mammary cells [4,5]. Exposure of MCF-7 human breast cancer cells to 17β-estradiol (E$_2$) activates transcription of the single-copy pS2 gene [6] and results in increased levels of pS2 mRNA and secreted protein [5,7–9]. Transient transfection assays have demonstrated that a single imperfect ERE confers estrogen-responsiveness to this gene [10].

The pS2 gene provides an ideal model system to study estrogen-regulated gene expression. The pS2 5' flanking region contains the elements of a classic estrogen-responsive gene including a TATA box, a CAAT box, and a single imperfect ERE [6,10]. In addition, expression of the pS2 gene has been used as a marker of estrogen responsiveness in ER-containing breast cancer cells, an indicator of disease progression, and a predic-

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tor of the success of antiestrogen therapy in breast cancer patients [9–13]. Thus, by examining the pS2 gene in MCF-7 cells, we can not only learn about general mechanisms involved in regulation of estrogen-responsive genes, but also gain specific insight into how the pS2 gene is regulated in human breast cancer cells.

Numerous studies aimed at delineating how estrogen-responsive genes are regulated have included the use of transient transfection assays. While these studies have provided us with a great wealth of information, transfection experiments have significant limitations. Cells transfected with receptor expression vectors typically contain much higher receptor levels than are found in normal target cells. This overexpression can lead to increased basal transcription and anomalous expression of reporter plasmids. Cells used in transfection assays may not contain accessory proteins needed for proper regulation and if present, these proteins may be present in limiting quantities. Multicopy reporter plasmids often contain synthetic promoters and sequences that bear little resemblance to native genes. Finally, the supercoiled plasmids used in transfection studies fail to take into account the role of native chromatin structure in regulating gene transcription.

To gain a better understanding of how endogenous, naturally occurring estrogen-responsive genes are regulated, we have used in vivo ligation mediated polymerase chain reaction (LMPCR) footprinting analysis to examine the 5′ flanking region of the endogenous estrogen-responsive pS2 gene in MCF-7 human breast cancer cells. By complementing these in vivo studies with in vitro footprinting and DNA binding assays, we have examined the role the ERE and the basal promoter in regulating pS2 gene expression and begun to define mechanisms by which estrogen and antiestrogens mediate their effects in target cells.

2. Materials and methods

2.1. Cell culture and ER preparations

MCF-7 (K1) cells [14] were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum and then transferred to MEM with 5% charcoal stripped [15] calf serum for 5 days and serum-free Improved MEM with 6 μg/ml transferrin, 0.25 mg/ml bovine serum albumin, 6 ng/ml insulin and 3.75 ng/ml hydrocortisone [16] 6 days before experiments were initiated. MCF-7 (K3) cells [14] were maintained in MEM with 5% charcoal stripped calf serum, harvested, and incubated with 10 nM E_2 for 20 min. Nuclear extracts were prepared as described [17], except that nuclei were extracted with buffer containing 0.5 M KCl. Viral stock for the production of ERα was kindly provided by J. Kadonaga and L. Kraus, University of California, San Diego, CA. ERα was expressed and purified as described by Kraus and Kadonaga [18].

2.2. Northern blot analysis

MCF-7 cells were exposed to control vehicle or 10 nM E_2 for 0.25–24 h. Total RNA was isolated using the Ultraspec RNA isolation system (Biotecx, Houston, TX) according to the manufacturer's instructions. A total of 10 μg of total RNA was fractionated on a 1.5% agarose gel and transferred to a nylon membrane. Hexamer primed ^{32}P-labeled pS2 and 36B4 [5,9] DNA fragments were used to probe the nylon membrane. Bands were visualized by autoradiography and quantitated using a phosphorimager and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

2.3. In vivo footprinting

For DNase I footprinting, MCF-7 cells, which had been treated with vehicle control or E_2 for 24 h, were permeabilized with 0.5% NP-40 for 3 min at 4°C or 0.2 mg/ml lysolecithin for 1 min at 25°C and treated with 750 U DNase I/ml. For dimethylsulfate (DMS) footprinting, MCF-7 cells, which had been treated with vehicle control or hormone for 2 h, were treated with 0.1% DMS for 2 min at 25°C. Isolation of genomic DNA and LMPCR footprinting was carried out essentially as described by Mueller and Wold [19]. A total of 1–2 μg genomic DNA was subjected to LMPCR using nested primers, which annealed to DNA sequences in the pS2 gene regions of interest. The linker primer oligos LMPCR1 and LMPCR2 described by Mueller and Wold [19] were also used. However, the first two 5′ nucleotides of LMPCR1 were omitted to limit secondary structure formation. The primers used to examine the pS2 ERE on the non-coding strand were: primer 1, 5′GGGATTACAGCGTGAGCCACTGC3′; primer 2, 5′AAAGAATTAGCTTAGGCCTAGACGGAATGG3′; and primer 3, 5′CTTAGGCCTAGACGGAATGGGCTTCAT3′. The annealing temperatures used for the primers were 60, 62, and 64°C, respectively. Excess primer 2 was removed using biotinlylated LMPCR1 and the Linker Tag Selection method [20]. Nested primers used to examine the basal promoter on the coding strand were: primer 4, 5′GGGCGCAGATCACCTTGTTC3′; primer 5, 5′GCCATTGCCTCCTCTCTGCTCC3′; and primer 6, 5′CCATTGCCTCCTCTCTGCTCCAAAGG3′. The annealing temperatures for these oligos were 56, 61, and 67°C, respectively.

2.4. Plasmid construction

To create pTZpS2($-666/+75$), a 741-bp DNA fragment containing sequence from the 5′ flanking re-

gion of the pS2 gene was synthesized by PCR amplification of -666 to $+75$ of the pS2 gene using sequence specific primers. The amplified, blunt-ended DNA fragment was inserted into Sma I-cut, dephosphorylated pTZ18U. Insert junctions were checked using DNA sequencing and the plasmids were purified on cesium chloride gradients.

2.5. In vitro DNase I footprinting

To examine the pS2 ERE, primers, which annealed 71 bp upstream (pS2for3 5′GCGCCAGG CCTA-CAATTTCATTATTAAAACCAA3′) and 87 bp downstream (pS2rev3 5′CAGGTCCTACTCATATCTGAG-AGGCCCTCCC3′) of the pS2 ERE were subjected to 30 rounds of PCR amplification with 30 ng of pTZpS2($-666/+75$) to produce 235-bp fragments. To examine the basal promoter elements, primers, which annealed 90 bp upstream (UpS2TATA.3 5′ATGT-AGCTTGACCATGTCTAGGAAACACCTTTGAT3′) and 45 bp downstream (primer 6) of the TATA sequence were subjected to 30 rounds of PCR amplification with 30 ng of pTZpS2($-666/+75$) to produce 203-bp DNA fragments. Labeling of the ERE- or TATA-containing DNA fragments was carried out with ^{32}P-labeled pS2rev3 or primer 6, respectively. The singly end-labeled amplified fragments were fractionated on an acrylamide gel and isolated. End-labeled DNA fragments (100 000 cpm) containing the pS2 ERE or basal promoter elements were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM MgCl$_2$, 50 ng of poly dI/dC and 4 mM DTT in a final volume of 50 μl with either 0–60 μg of nuclear extract from E$_2$-treated MCF-7 cells or 0.6–2.4 pmol of purified flag-tagged, E$_2$-occupied ER. Bovine serum albumin or ovalbumin was included in the binding reaction so that the total protein concentration in each reaction was 2.5 μg for purified ER or 60 μg for nuclear

extracts. Poly dI/dC was increased to 1 μg per reaction when nuclear extracts were used. A total of 1–2 U of RQ1 ribonuclease-free DNase I (Promega, Madison, WI) was added to each sample and incubated at room temperature for 0.75–8 min. The DNase I digestion was terminated by addition of stop solution (200 mM NaCl, 1% SDS, 30 mM EDTA and 100 ng/μl tRNA). DNA was phenol/chloroform extracted, precipitated, fractionated on an 8% denaturing acrylamide gel. Radioactive bands were visualized by autoradiography.

2.6. Gel mobility shift assays

Gel mobility shift assays were carried out essentially as described [21,22]. ^{32}P-labeled (10 000 cpm) 235-bp DNA fragments containing the pS2 ERE were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 50 ng of poly dI/dC and 4 mM DTT in a final volume of 20 μl with either 10 μg of MCF-7 nuclear extract or 90 fmol of purified ER. BSA was included when purified ER was used so that the total protein concentration in each reaction was 2.5 μg. When MCF-7 nuclear extracts were used, the non-specific DNA for each reaction included 1 μg of salmon sperm DNA and 2 μg poly dI/dC. For antibody supershift experiments, the ER-specific monoclonal antibody, h151 (kindly provided by Dean Edwards, University of Colorado, Denver, CO) was added to the protein-DNA mixture and incubated for 5 min at room temperature. Low ionic strength gels and buffers were prepared as described [23]. Radioactive bands were visualized by autoradiography.

3. Results

3.1. Estrogen treatment of MCF-7 cells increases pS2 mRNA levels

In order to limit basal expression of the pS2 gene, MCF-7 breast cancer cells were maintained on serum free medium for 6 days and then exposed to either control vehicle or E$_2$ for 0.25–24 h. The 10-nM E$_2$ concentration used in these studies has been shown to fully occupy the receptor and maximally stimulate pS2 gene expression [24]. Basal and E$_2$-induced pS2 mRNA levels present in MCF-7 cells were determined using Northern blot analysis. Increased levels of pS2 mRNA were detected after a 1-h exposure of cells to E$_2$ and continued to increase up to 24 h (Fig. 1). In contrast, the level of constitutively expressed 36B4 mRNA [9], which was used as an internal control, remained constant. Quantitation from four independent determinations demonstrated that pS2 mRNA transcripts increased 16-fold after 24 h of E$_2$ treatment. These
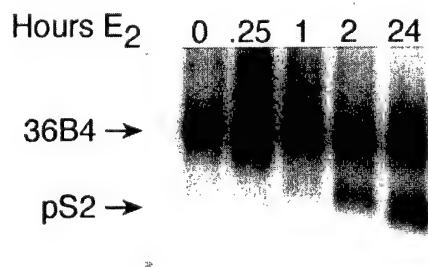
Fig. 1. Effects of E$_2$ on pS2 mRNA production in MCF-7 breast cancer cells assessed by Northern blot analysis. Total RNA was isolated from MCF-7 cells that had been treated with E$_2$ for varying periods of time, fractionated on an agarose gel, and transferred to a nylon membrane. ^{32}P-labeled pS2 and 36B4 probes were used to detect the mRNAs. Equivalency of mRNA loading was monitored by normalization to levels of constitutively-expressed 36B4.
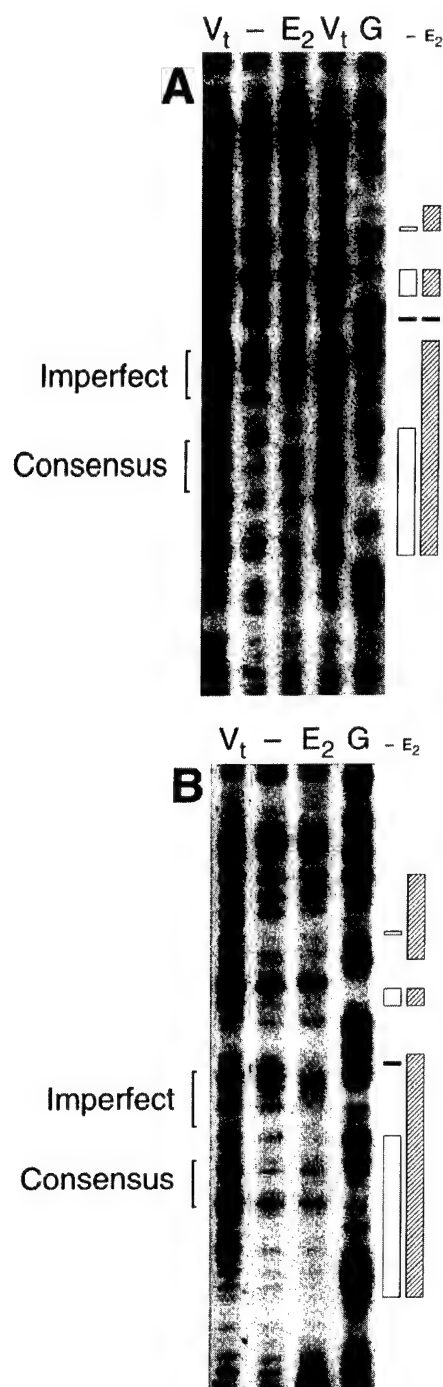
Fig. 2. In vivo DNase I footprinting of the pS2 ERE. MCF-7 cells were exposed to control vehicle (−) or E_2 and then treated with either NP-40/DNase I (panel A) or lysolecithin/DNase I (panel B). Genomic DNA was isolated and used for in vivo LMPCR footprinting. Naked genomic DNA samples, which had been treated in vitro with either DNase I (V_t) or DMS (G), were included as references. Nucleotides protected in control (open bars) or E_2 (hatched bars) treated cells are indicated to the right of the autoradiogram. Solid bars indicate regions of DNase I hypersensitivity.

findings are in good agreement with previous determinations [8–10,24] and indicate that the pS2 gene was essentially quiescent in the absence of exogenously

added E_2 but responded robustly to E_2 treatment. Thus, we were assured that the serum-free conditions utilized in our studies fully supported pS2 gene expression.

3.2. In vivo DNase I footprinting reveals protection of the endogenous pS2 ERE

To define how the ERE is involved in regulating endogenous target genes in living cells, we used in vivo DNase I footprinting to examine the endogenous pS2 ERE residing in native chromatin in MCF-7 cells. This technique utilizes the non-specific cleavage properties of DNase I to identify DNA regions that are protected by proteins. The pS2 ERE is located from − 393 to − 405 relative to the transcription initiation site and is comprised of a 5′ consensus ERE half site and a 3′ imperfect ERE half site (5′GGTCAnnnTGGCC3′; [10]).

MCF-7 cells were treated with ethanol vehicle or E_2, harvested, and then exposed to an NP40/DNase I mixture to cleave accessible DNA sequences that were not protected by proteins. Cells were lysed, DNA was isolated, and LMPCR procedures were carried out. Isolated, naked genomic DNA was treated with DNase I in vitro and used as a reference to identify DNA sequences that were susceptible to cleavage in the absence of proteins. A comparison of in vitro- and in vivo-cleaved genomic DNA revealed that the consensus ERE half site and adjacent sequences were protected when MCF-7 cells were maintained in a hormone-free environment (Fig. 2A, compare V_t and −). Regions of protection (open bars) and DNase I hypersensitivity (solid bar) were also observed in the absence of hormone. When MCF-7 cells were treated with E_2 and subjected to in vivo DNase I footprinting procedures, regions of protection (Fig. 2A, E_2, hatched bars) and enhanced cleavage (solid bar) were similar to those observed in the absence of hormone. However, the protection at the ERE was extended to include the imperfect ERE half site and adjacent nucleotide sequence.

As a comparison, in vivo DNase I footprinting experiments were also carried out using MCF-7 cells that had been treated with a lysolecithin/DNase I mixture. When in vitro-treated DNA was compared to in vivo-treated DNA, nucleotides within and adjacent to the consensus ERE half site were protected in the absence of hormone (Fig. 2B, compare V_t and −, open bar). DNase I hypersensitivity and regions of protection were also observed adjacent to the imperfect ERE half site. Exposure of MCF-7 cells to E_2 resulted in a more extensive pattern of protection than was observed in the absence of hormone. Both the imperfect and consensus ERE half sites were protected and nucleotides adjacent to the imperfect ERE half site were more extensively protected when cells were exposed to E_2 (Fig. 2B, E_2, hatched bars).

Similar footprinting patterns were observed in the region of the pS2 ERE in four to five independent LMPCR experiments using two sets of MCF-7 cells that had been permeabilized with either NP40 or lysolecithin. Although some variation in individual nucleotides protected was detected, which probably resulted from slightly different amounts of DNase I entry into the cells, it was clear that the overall observations were quite similar. The consensus ERE half site was protected in the absence of hormone, both ERE half sites were occupied in the presence of E_2, and sites adjacent to the imperfect ERE half site were more extensively protected after E_2 treatment.

3.3. DMS footprinting delineates nucleotides involved in modulating pS2 expression

In order to obtain additional information about proteins interacting with the pS2 5' flanking region, MCF-7 cells were treated with control vehicle or E_2 and then exposed to DMS in order to methylate individual guanine residues that were not intimately associated

Fig. 3. In vivo DMS footprinting of the pS2 ERE. MCF-7 cells were exposed to control vehicle (−), 10 nM E_2 (E_2), 100 nM 4-hydroxytamoxifen (T), or 100 nM ICI 182,780 (I) and then treated with DMS. Genomic DNA was isolated and used in in vivo LMPCR footprinting. A naked genomic DNA sample, which had been treated in vitro with DMS (G), was included for reference. Nucleotides protected in control (open bars), E_2 (hatched bars), 4-hydroxytamoxifen (crosshatched) or ICI 182,780 (striped bars) treated cells are indicated to the right of the autodiogram. Solid bars indicate regions of enhanced cleavage.

with proteins. Cells were lysed, DNA was isolated, methylated guanines were cleaved with piperidine, and LMPCR procedures were carried out. Distinct differences were apparent in the footprinting patterns when MCF-7 cells had or had not been exposed to E_2. When MCF-7 cells were cultured in a hormone-free environment, the footprinting pattern observed was very similar to that of in vitro DMS-treated naked, genomic DNA, except that three adenine residues, one of which was located in the consensus ERE half site, displayed an increased sensitivity to DMS methylation (Fig. 3, compare G and –). Enhanced DMS cleavage of adenine residues can result from binding of a protein to the major groove of the DNA helix and/or distortion of the DNA helix causing adenine residues in the minor groove to become more accessible to DMS treatment. Only one guanine residue in this region appeared to be protected in the absence of hormone.

When cells were exposed to E_2, one guanine residue in the imperfect ERE half site was protected and the adenine residue in the consensus ERE half site displayed increased sensitivity to DMS methylation (Fig. 3, E_2), as was observed in the absence of hormone. Even more striking was that the pattern of protection extended to include sequences flanking both sides of the ERE and multiple regions adjacent to the imperfect ERE half site. Enhanced adenine and guanine cleavage was also observed. These findings reinforced the idea that E_2 was not only affecting the interaction of protein(s) with the ERE, but multiple other protein-DNA interactions as well, particularly in regions adjacent to the imperfect ERE.

3.4. Treatment of MCF-7 cells with antiestrogens elicits unique footprinting patterns

A subject of great clinical interest has been to delineate how tamoxifen, an antiestrogen with agonistic and antagonistic properties, and ICI 182,780, a pure antiestrogen, inhibit disease recurrence in breast cancer patients [25–27]. Although 4-hydroxytamoxifen has a weak agonistic effect on pS2 mRNA levels, ICI 182,780 does not increase pS2 RNA levels [24,28,29]. The effects of these compounds have been studied using in vitro DNA binding assays and transient transfection assays, but their effects on protein-DNA interactions at the molecular level in a native gene have not been addressed.

When MCF-7 cells were treated with 4-hydroxytamoxifen, the footprinting pattern observed was strikingly similar to that of in vitro DMS-treated naked genomic DNA, except that a guanine residue in the consensus ERE half site (Fig. 3, T, cross hatched bars) and two more distant regions 3' of the ERE were strongly protected. Thus, treatment of MCF-7 cells with 4-hydroxytamoxifen resulted in minimal changes
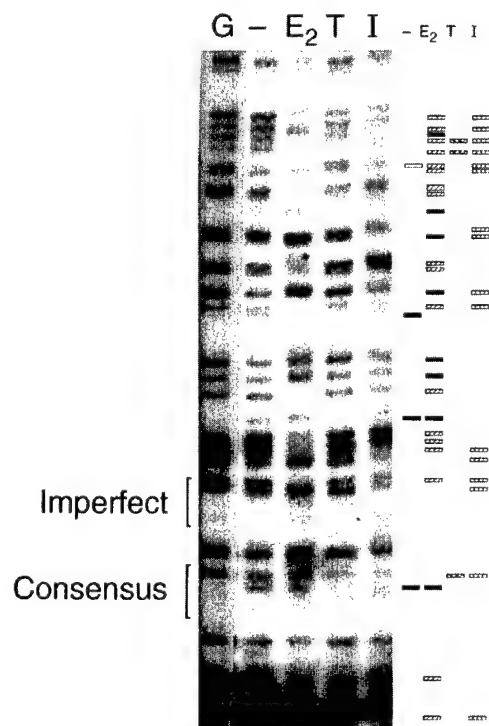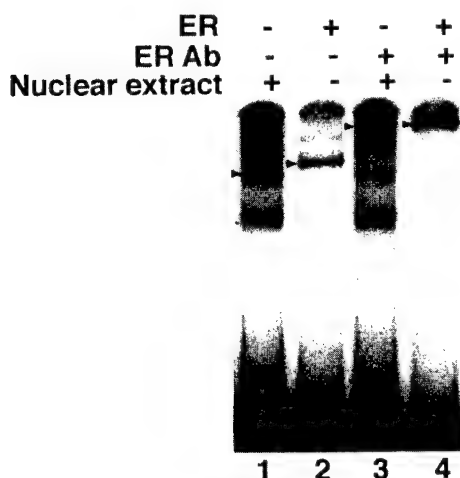
Fig. 4. Binding of MCF-7 and purified ER to the ERE-containing DNA fragments. The 235-bp ^{32}P-labeled DNA fragments containing pS2 5' flanking region from −505 to −270 were incubated with nuclear extracts from E$_2$-treated MCF-7 cells (lanes 1 and 3) or purified ER (lanes 2 and 4). The ER-specific antibody h151 (ER Ab) was added to the binding reaction as indicated. The ^{32}P-labeled oligos were fractionated on a non-denaturing gel and visualized by autoradiography. Arrowheads (▶) indicate ER-containing DNA complexes.

in the protection of this region of the pS2 5' flanking region.

When MCF-7 cells were treated with ICI 182,780, a very different and distinct footprinting pattern was observed. Guanine residues in the consensus (Fig. 3, I, striped bars) and imperfect ERE half sites and adjacent nucleotide sequence were protected. Numerous changes in protein-DNA interactions were also observed at multiple sites adjacent to the imperfect ERE half site. Thus, the two antiestrogens tested, one a partial agonist/antagonist and the other a pure antagonist, produced very different footprinting patterns.

3.5. The ER and other proteins interact with the pS2 promoter in vitro

In vivo footprinting is a powerful technique which can identify cis elements involved in mediating changes in transcription. However, it cannot be used to identify factors bound to these elements. Thus, a series of in vitro experiments was carried out to characterize the interaction of proteins with the pS2 promoter. Since ERα is present at high levels in MCF-7 cells [14], but ERβ is not expressed [30], it seemed likely that ERα was responsible for the protection of the pS2 ERE seen in our in vivo footprinting experiments. To determine if this was the case, gel mobility shift experiments were carried out. When ^{32}P-labeled DNA fragments, each comprised of 235 bp of pS2 of 5' flanking region (− 505 to − 270), were incubated with MCF-7 nuclear extracts, several gel-shifted bands were observed (Fig. 4, lane 1). In contrast, when purified, flag-tagged ERα

(hereafter referred to as ER) was used, a single gel-shifted band was present (lane 2). Addition of the ER-specific antibody h151 to the binding reaction containing nuclear extract resulted in the disappearance of a single band (lane 1, ▶), which migrated slightly faster than the purified ER-DNA complex (lane 2, ▶), and the appearance of a more slowly migrating, supershifted band (lane 3, ▶). As expected, the ER-specific antibody efficiently supershifted the receptor-DNA complex (lane 4, ▶). These findings indicate that the protein-DNA complex, which migrated slightly faster than the purified ER-DNA complex, contained the MCF-7 ER. The difference in migration of the MCF-7 ER-DNA complex and the purified ER-DNA complexes most likely resulted from the slightly larger size of the flag-tagged, purified ER or minimal proteolytic cleavage of the MCF-7 ER. These experiments demonstrate that the ER from MCF-7 nuclear extracts and purified ER bind to the region of the pS2 promoter containing the ERE.

In vitro DNase I footprinting was carried out to determine if the pS2 ERE was protected by protein present in nuclear extracts of E$_2$-treated MCF-7 cells. When ^{32}P-labeled DNA fragments containing 235 bp from − 505 to − 270 of the pS2 5' flanking sequence were combined with increasing amounts of nuclear extract, the consensus ERE half site was more extensively protected than the imperfect ERE half site and regions adjacent to the imperfect ERE half site were protected (Fig. 5). Several regions of hypersensitivity were also observed. Experiments carried out with nuclear extracts from MCF-7 cells that had been treated with ethanol vehicle produced identical footprints (data not shown). Kraus and Kadonaga [18] have demonstrated that when an ERE-containing DNA template, which does not respond to hormone in vitro, is assembled into nucleosomes, it regains estrogen responsiveness suggesting that appropriately structured DNA is required for hormone-regulated gene expression in vivo and in vitro.

To determine whether the ER was able to bind to the ERE in the absence of other MCF-7 nuclear proteins, in vitro DNase I footprinting experiments were also carried out with E$_2$-occupied, purified ER. The 235-bp ^{32}P-labeled DNA fragments containing the pS2 ERE and flanking sequences were incubated with increasing concentrations of baculovirus-expressed, purified ER. As increasing amounts of purified ER were added to the binding reaction, an incremental increase in protection of the pS2 ERE was observed (Fig. 6). The consensus ERE half site was protected at lower ER concentrations than the imperfect ERE half site demonstrating the receptor's preference for the consensus ERE half site. Regions flanking both sides of the ERE displayed increased hypersensitivity to DNase I cleavage. The region adjacent to the consensus ERE half site was particularly sensitive to DNase I cleavage.

3.6. The pS2 basal promoter is poised for transcription even in the absence of hormone

All our in vivo and in vitro assays supported the idea that the pS2 ERE is instrumental in regulation of the pS2 gene. However, the ERE does not function in isolation, but requires the participation of other cis elements for regulated gene expression. Since previous
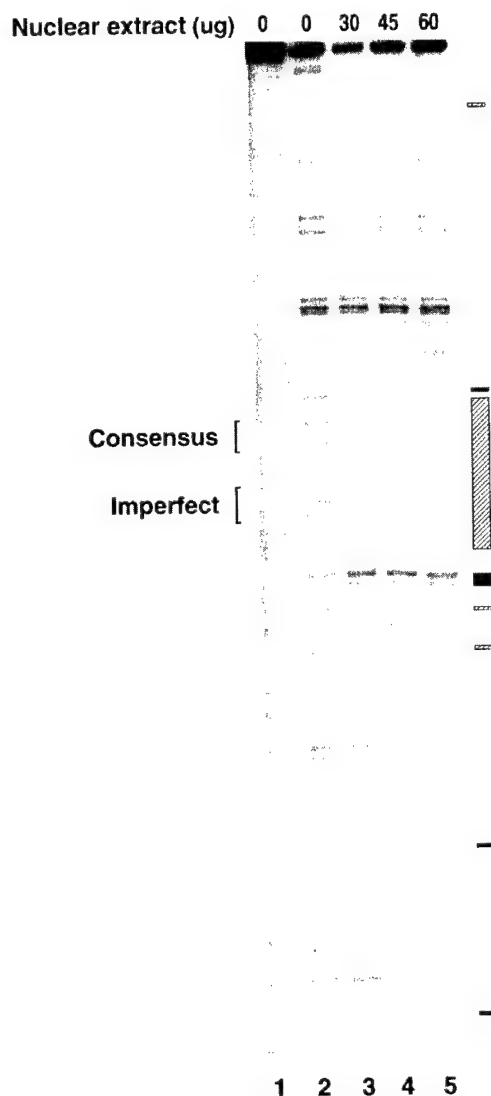

Fig. 5. In vitro DNase I footprinting of the pS2 ERE with MCF-7 nuclear extracts. The 235-bp ^{32}P-labeled DNA fragments containing pS2 5′ flanking region from −505 to −270 were incubated with increasing concentrations of nuclear extract from E_2-treated MCF-7 cells (lanes 3–5). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments cleaved in vitro with DMS (lane 1) or DNase I (lanes 2) in the absence of proteins were included as references. The locations of the consensus and imperfect ERE half sites are indicated. Protected (hatched bars) and hypersensitive (solid bars) nucleotides are indicated to the right of the autoradiogram. Because of the labeling procedures used, in vivo and in vitro footprinting patterns are in opposite orientations.

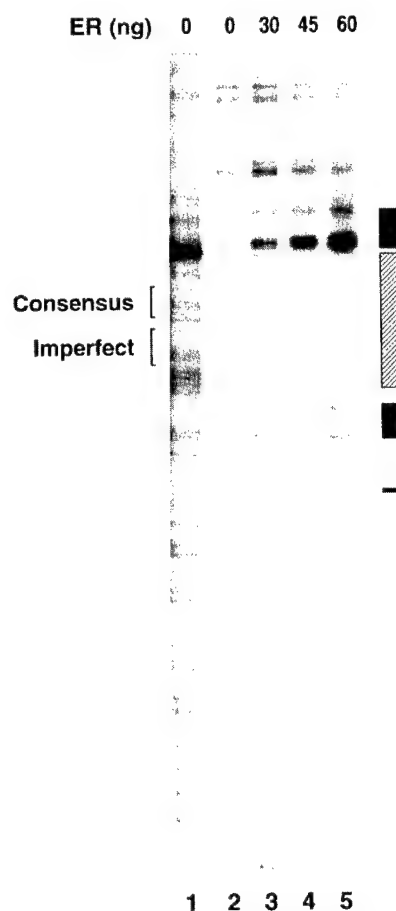Fig. 6. In vitro DNase I footprinting of the pS2 ERE with purified ER. The 235-bp ^{32}P-labeled DNA fragments containing pS2 5′ flanking region from −505 to −270 were incubated with increasing concentrations of purified E_2-occupied ER (lanes 3–5). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments cleaved in vitro with DMS (lane 1) or DNase I (lanes 2) in the absence of proteins were included as references. The locations of the consensus and imperfect ERE half sites are indicated. Protected (hatched bar) and hypersensitive (solid bars) regions are indicated to the right of the autoradiogram. Because of the labeling procedures used, in vivo and in vitro footprinting patterns are in opposite orientations.

studies have suggested that proteins bound to the ER interact directly or through adapter proteins with transcription factors bound to the basal promoter in order for transcription to occur [31–34], we were particularly interested in examining whether hormone treatment affected the interaction of proteins with the basal promoter. Surprisingly, the footprinting patterns in the TATA and CAAT regions of the pS2 gene were nearly identical when MCF-7 cells were treated with either control vehicle or E_2 and then exposed to DNase I (Fig. 7). Two small regions of protection and two extended regions of protection were observed before and after E_2 treatment (hatched bars). Interestingly, multiple DNase I hypersensitive sites were observed flanking the TATA

and CAAT sequences in both control vehicle- and E_2-treated cells. The presence of hypersensitive sites in these regions suggests that protein-induced conformational changes brought about by binding of transcription factors to these regions may enhance the susceptibility of specific nucleotides to DNase I cleavage [35].

This basal promoter region was also examined using in vitro DNase I footprinting. A 203-bp ^{32}P-labeled DNA fragment containing the pS2 promoter from − 154 to + 49 was incubated with nuclear extracts from E_2-treated MCF-7 cells. As seen in the in vivo footprinting experiments, numerous hypersensitive regions flanked both sides of the TATA and CAAT sequences (Fig. 8). Regions of protection were also observed and were particularly evident in the region between the TATA and CAAT sequences.
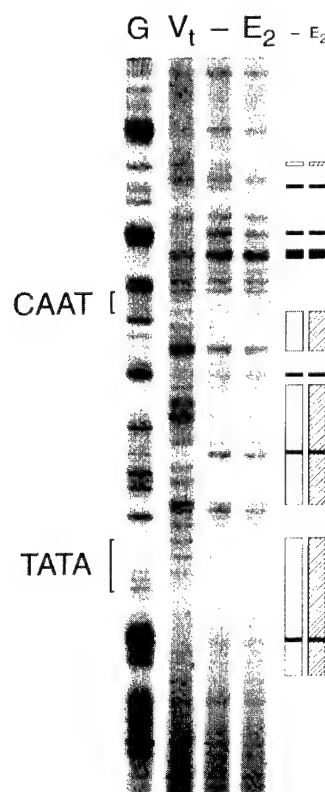

Fig. 7. In vivo DNase I footprinting of the basal promoter. MCF-7 cells were treated with control vehicle (−) or 10 nM E_2 (E_2) and then exposed to NP40/DNase I. Genomic DNA was isolated and used in in vivo LMPCR footprinting. Naked genomic DNA samples, which had been treated in vitro with either DNase I (V_t) or DMS (G), were included as references. Nucleotides protected in control- (open bars) or E_2- (hatched bars) treated cells are indicated to the right of the autoradiogram. Solid bars indicate regions of DNase I hypersensitivity.
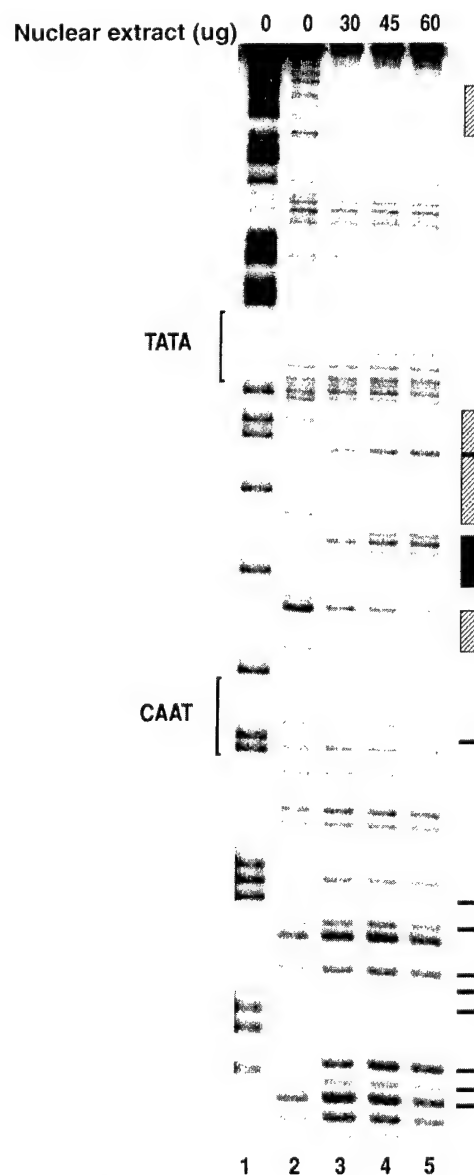

Fig. 8. In vitro DNase I footprinting of the pS2 basal promoter with MCF-7 nuclear extracts. The 203-bp ^{32}P-labeled DNA fragments containing pS2 5′ flanking region from − 154 to + 49 were incubated with increasing concentrations of nuclear extract from E_2-treated MCF-7 cells (lanes 3–5). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments cleaved in vitro with DMS (lane 1) or DNase I (lanes 2) in the absence of proteins were included as references. The locations of the TATA and CAAT sequences are indicated. Protected (hatched bars) and hypersensitive (solid bars) nucleotides are indicated to the right of the autoradiogram. Because of the labeling procedures used, in vivo and in vitro footprinting patterns are in opposite orientations.

4. Discussion

A number of methods have been employed to examine regulation of the estrogen-responsive pS2 gene. Extensive studies carried out by Chambon and coworkers have defined the effects of estrogen treatment on the

synthesis of pS2 mRNA and delineated specific sequences in the pS2 5′ flanking region involved in regulating gene expression [5,7–10,28]. These studies have been extremely informative and have formed the basis of our current understanding of how the pS2 gene is regulated in breast cancer cells. However, in a study comparing the expression of the endogenous pS2 gene and a transiently transfected reporter plasmid containing 1.1 kb of the pS2 5′ flanking region, the endogenous pS2 gene residing in native chromatin responded differently to antiestrogen treatment than the transiently transfected pS2 promoter residing in a supercoiled reporter plasmid [24]. These findings indicate that DNA context may be important for the appropriate regulation of the pS2 gene and that chromatin structure may exert an additional level of control on regulation of this gene.

4.1. Role of the ERE in estrogen-regulated pS2 gene expression

To more fully understand how the endogenous estrogen-responsive pS2 gene is regulated, we have used high resolution in vivo footprinting to examine the interaction of proteins with the endogenous pS2 5′ flanking region residing in native chromatin. We found that when MCF-7 cells were treated with E_2, the ERE and regions flanking this sequence were extensively protected using both DNase I and DMS in vivo footprinting analysis. DNase I footprinting revealed that the consensus ERE half site was protected in the absence of hormone and that both ERE half sites and adjacent flanking sequences were protected after hormone treatment. The enhanced DMS sensitivity of an adenine residue in the consensus ERE half in the absence and in the presence of E_2 and the protection of a guanine residue in the imperfect ERE half site in the presence of E_2 further support the DNase I footprinting results.

Our in vivo footprinting analysis complemented by our in vitro binding studies, supports the idea that unoccupied ER is bound to the consensus ERE half site in the absence of hormone and that an E_2-occupied ER is bound to both ERE half sites in the presence of hormone. Since we do observe protection of nucleotide sequence adjacent to the consensus ERE half site in the absence of hormone in our in vivo footprints, it is possible that ER binding to the consensus ERE half site could be stabilized by interaction with a protein bound to adjacent nucleotide sequence. The ability of the receptor to bind to the consensus ERE half site in the absence of hormone could also be fostered by a rather loose association of the ERE-containing DNA with histones [36]. Taken together, our findings imply that differential occupation of the ERE may be involved in silencing, activation, and maintenance of pS2 gene expression.

It is interesting to note that hypersensitive sites are present in our in vivo and in our in vitro footprints. Such hypersensitivity may result from distortion or bending of the DNA helix by protein binding. We and others have demonstrated that the ER induces distortion and directed bending in ERE-containing DNA fragments [37–40]. Binding of ER to the ERE could feasibly explain the hypersensitivity observed in this region.

Our findings contrast with those of the apo very low density lipoprotein 5′ flanking region in which the EREs were occupied only after estrogen treatment [41,42]. However, studies of a number of hormone-responsive genes indicate that there is significant variation in the protection of hormone response elements. While some response elements are occupied only in the presence of hormone [43–47], others appear to be unaffected by hormone treatment [46,48–50]. These apparent differences in the occupation of various hormone response elements may be due to the presence of tissue-specific accessory factors, the inaccessibility of protein binding sites due to promoter organization, or the transient nature of protein-DNA interactions.

4.2. Mechanisms regulating antiestrogen action

Tamoxifen, a non-steroidal antiestrogen, has been extensively used in breast cancer therapy and is also being tested for its ability to decrease the onset of breast cancer [51]. ICI 182,780, an estradiol analogue, has also been used in breast cancer treatment and may prove to be useful in limiting disease recurrence in tamoxifen-resistant tumors [52]. While tamoxifen has both agonistic and antagonistic actions [26,53,54] and moderately enhances pS2 mRNA levels in MCF-7 cells [24,28], ICI 182,780 is classified as a pure antiestrogen [55] and does not induce pS2 mRNA levels in MCF-7 cells [29].

Antiestrogens have had a tremendous impact on breast cancer treatment and yet, the mechanisms by which they bring about their effects are largely unknown. Our in vivo footprinting experiments provide us with a first glimpse of how these compounds function at the level of the gene and demonstrate that 4-hydroxytamoxifen and ICI 182,780 have very different effects on the interaction of proteins with the pS2 5′ flanking region. Although few proteins were recruited to the pS2 5′ flanking region after 4-hydroxytamoxifen treatment of MCF-7 cells, multiple proteins were recruited after treatment of MCF-7 cells with ICI 182,780. The different patterns of protein-DNA interaction observed after treatment of cells with 4-hydroxytamoxifen and ICI 182,780 may underlie the partial agonist/antagonist and pure antagonist properties, respectively, of these two drugs.

4-Hydroxytamoxifen and ICI 182,780 produced very different footprints, which were distinct from the footprints observed with no hormone or E_2 treatment. What is unclear at this point is how such differences in footprinting patterns are brought about. Part of the answer may lie in the changes in ER conformation that accompany binding of hormone or antihormone. A number of studies have provided evidence that individual ligands may induce specific changes in ER conformation [56–58]. Recent X-ray crystallographic studies of the estrogen and antiestrogen-bound ER ligand binding domain demonstrate that there are dramatic differences in the orientation of helix 12 when the ligand binding domain is occupied by E_2 or raloxifene [59]. Such changes in receptor conformation could result in the presentation of different functional ER surfaces and form the basis for the recruitment of specific sets of transcription factors to the promoter.

4.3. Role of the basal promoter in transcription activation

A previous in vitro transcription study suggested that ER binding to the ERE enhanced transcription by stabilizing binding of proteins to the basal promoter [60]. Surprisingly, however, elements in the endogenous pS2 basal promoter appeared to be largely unaffected by E_2 treatment in our in vivo footprinting studies. There was no evidence of enhanced protection or altered hypersensitivity in this region after E_2 treatment suggesting that the basal promoter is accessible and poised for transcription even in the absence of hormone. In support of this idea, Sewack and Hansen [36] have reported that the nucleosome containing the TATA sequence is not altered by E_2 treatment and that the loose association of this nucleosome with the histone octamer may allow protein complex formation in the presence and in the absence of hormone. Thus, it appears that recruitment and binding of transcription factors to the basal promoter does not play a role in modulating transcription of the pS2 gene.

The pS2 TATA and CAAT sequences were flanked by hypersensitive sites before and after hormone treatment. Since the TATA binding protein binds to the minor groove of the DNA helix and induces DNA to bend [61], this increased sensitivity to DNase I may result from distortion of the DNA helix brought about by binding of the TATA binding protein and other factors associated with the basal transcription complex.

4.4. Role of other proteins in estrogen-regulated transcription activation

Although estrogen treatment did not result in recruitment of proteins to the basal promoter, it did play a substantial role in recruitment and binding of proteins to other regions of the pS2 5′ flanking region, in particular, those regions within the ERE and adjacent to the imperfect ERE half site. Our in vivo and in vitro footprinting experiments with MCF-7 nuclear extracts demonstrated that several sites flanking the ERE were protected by proteins after E_2 treatment providing evidence that a number of proteins intimately associated with the pS2 promoter play an integral role in regulating gene expression.

Numerous groups have reported the association of steroid hormone receptors with coactivators and corepressors (reviewed in [62] and references therein). Recent studies have also identified coactivator and corepressor proteins with histone acetylase and deacetylase activities, respectively [63–66]. Association of ER with these coregulators may be important in modulating the accessibility of transcription factor binding sites in native chromatin. The divergent footprinting patterns observed with estrogen- and antiestrogen-treated MCF-7 cells suggest that unoccupied, estrogen-occupied, and antiestrogen-occupied ER associate with different sets of coactivator and/or corepressor proteins and that these proteins may in turn form an interconnected protein-DNA complex. Estrogen treatment could release corepressor proteins and promote interaction of the receptor with coactivators. Taken together, our in vivo and in vitro experiments provide us with a more physiologically relevant view of how estrogens and antiestrogens regulate the expression of estrogen-responsive genes in target cells.

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Sp1 Binding Sites and An Estrogen Response Element Half-Site Are Involved in Regulation of the Human Progesterone Receptor A Promoter

APPENDIX 2

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Progesterone receptor gene expression is induced by estrogen in MCF-7 human breast cancer cells. Although it is generally thought that estrogen responsiveness is mediated through estrogen response elements (EREs), the progesterone receptor gene lacks an identifiable ERE. The progesterone receptor A promoter does, however, contain a half-ERE/Sp1 binding site comprised of an ERE half-site upstream of two Sp1 binding sites. We have used *in vivo* deoxyribonuclease I (DNase I) footprinting to demonstrate that the half-ERE/Sp1 binding site is more protected when MCF-7 cells are treated with estrogen than when cells are not exposed to hormone, suggesting that this region is involved in estrogen-regulated gene expression. The ability of the half-ERE/Sp1 binding site to confer estrogen responsiveness to a simple heterologous promoter was confirmed in transient cotransfection assays. *In vitro* DNase I footprinting and gel mobility shift assays demonstrated that Sp1 present in MCF-7 nuclear extracts and purified Sp1 protein bound to the two Sp1 sites and that the estrogen receptor enhanced Sp1 binding. In addition to its effects on Sp1 binding, the estrogen receptor also bound directly to the ERE half-site. Taken together, these findings suggest that the estrogen receptor and Sp1 play a role in activation of the human progesterone receptor A promoter. (Molecular Endocrinology 14: 972–985, 2000)

INTRODUCTION

Estrogen is a hormone of critical importance in the development and maintenance of reproductive tissues and also plays an important role in cardiovascular and bone physiology. Estrogen's effects are mediated through its interaction with the intracellular estrogen receptor (ER). Numerous studies have demonstrated that the two ERs, α and β, mediate their effects by binding to specific DNA sequences, estrogen response elements (EREs), thereby initiating changes in transcription of target genes (1, 2).

It has become apparent that, in addition to binding directly to an ERE, the ER may also modulate transcription indirectly by interacting with other DNA-bound proteins. For example, ER interaction with AP1-bound fos and jun proteins confers estrogen responsiveness to the ovalbumin (3), c-*fos* (4), collagenase (5), and insulin-like growth factor I (6) genes. In addition, a growing body of evidence suggests that the ER may influence binding of Sp1 to its recognition site and thereby confer estrogen responsiveness to the creatine kinase B (7), c-*myc* (8), retinoic acid receptor α (9), heat shock protein 27 (10, 11), cathepsin D genes (12), and uteroglobin (13) genes.

The progesterone receptor (PR) gene is under estrogen control in normal reproductive tissues (14, 15) and in MCF-7 human breast cancer cells (16, 17). MCF-7 PR mRNA and protein increase and reach maximal levels after 3 days of 17β-estradiol (E$_2$) treatment (16–18). Like ER, two distinct PR forms are differentially expressed in a tissue-specific manner (19–23). PR-B is a 120-kDa protein containing a 164 amino acid amino-terminal region that is not present in the 94-kDa PR-A. Two discrete promoters, A and B, which are thought to be responsible for the production of PR-A and PR-B, respectively, have also been defined (24). The activities of these two promoters are increased by estrogen treatment of transiently transfected Hela cells. Interestingly, no consensus EREs have been identified in either promoter A (+464 to +1105) or promoter B (−711 to +31). Promoter A does, however, contain an ERE half-site located upstream of two Sp1 sites (24). The presence of these adjacent binding sites suggests that the ER might be able to influence PR expression directly by binding to the ERE half-site, indirectly by interacting with proteins bound to the putative Sp1 sites, or a combination of
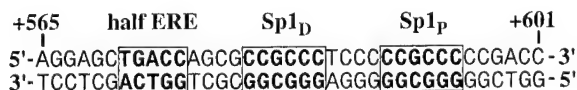
these two methods. To determine whether the ERE half-site and the two Sp1 sites present in the human PR-A promoter might impart estrogen responsiveness to the PR gene, a series of *in vivo* and *in vitro* experiments were carried out.

RESULTS

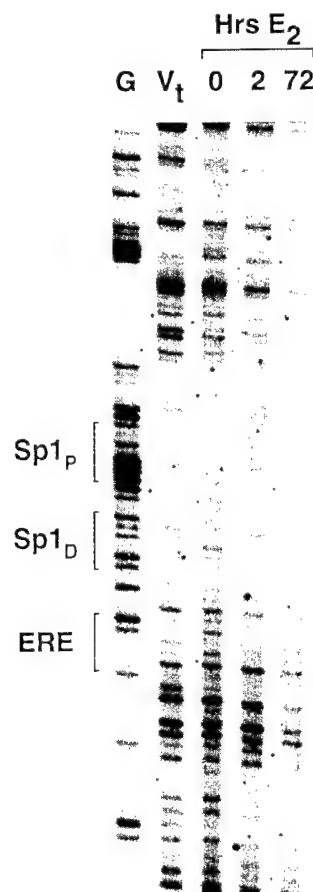### *In Vivo* Footprinting of the PR Gene

A number of studies have suggested that an Sp1 site alone or in combination with an imperfect ERE or ERE half-site may be involved in conferring estrogen responsiveness to target genes (7–13). To determine whether the ERE half-site and two potential Sp1 sites residing in the endogenous human PR gene (+571 to +595, Ref. 24) might be involved in estrogen-regulated transactivation, *in vivo* deoxyribonuclease I (DNase I) footprinting was carried out using MCF-7 cells. The region of the PR-A promoter containing the consensus ERE half-site and two potential Sp1 sites is shown in Fig. 1 and will hereafter be referred to as the half-ERE/Sp1 binding site.

To carry out *in vivo* footprinting assays, MCF-7 cells were treated with ethanol vehicle or with E_2 for 2 or 72 h and then exposed to DNase I. The cells were rapidly lysed, DNA was isolated, and ligation-mediated PCR (LMPCR) procedures were carried out (25). Naked genomic DNA, which had been treated *in vitro* with DNase I, served as a reference in identifying sequences that were susceptible to cleavage in the absence of proteins (Fig. 2, V_t). When cells were treated with E_2 for 2 h, the protection of the proximal Sp1 site ($Sp1_P$), the distal Sp1 binding site ($Sp1_D$), and the ERE half-site was greater than seen in cells that had not been exposed to hormone. After 72 h of E_2 treatment, a time when PR mRNA and protein reach maximal levels (16–18, 26, 27), the protection of the half-ERE/Sp1 binding site was sustained. E_2 treatment also elicited protection of regions flanking the half-ERE/Sp1 binding site. Thus, we were able to detect distinct differences in protection of the half-ERE/Sp1 binding site on the coding strand of the endogenous PR gene after E_2 treatment. Despite numerous attempts, we were unable to obtain a footprint of the noncoding PR DNA strand in this region. The failure of these LMPCR reactions may have been due to formation of an extensive stem loop structure ($\Delta G = -11.5$ kcal/mol)

Fig. 2. *In Vivo* DNase I Footprinting of the Endogenous PR Gene in MCF-7 Cells

MCF-7 cells were maintained in serum-free medium for 5 days, treated with ethanol control (0 h E_2) or 1 nM E_2 for 2 or 72 h, and then exposed to DNase I. Genomic DNA was isolated and used in *in vivo* footprinting. Naked genomic DNA, which had been treated *in vitro* with either DMS (G) or DNase I (V_t), was included as a reference. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$), and ERE half-site are indicated.

extending from +674 to +733 (24) that limited primer annealing or interfered with the ability of polymerase to proceed through this region. Nonetheless, our *in vivo* footprinting of the coding strand demonstrated that the half-ERE/Sp1 binding site residing in the endogenous PR gene was differentially protected in ethanol- and E_2-treated MCF-7 cells and suggested that the ERE half-site as well as the proximal and distal Sp1 sites might be involved in regulation of the endogenous PR gene in MCF-7 cells.

Estrogen Enhances Transcription of a Reporter Plasmid Containing the Half-ERE/Sp1 Binding Site
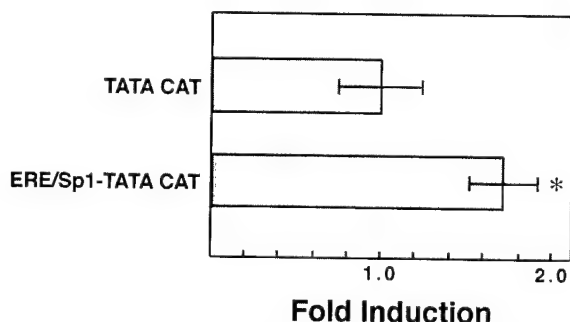
To determine whether the half-ERE/Sp1 binding site could confer estrogen responsiveness to a heterolo-

```
+565      half ERE     Sp1_D        Sp1_P      +601
 |                                               |
5'-AGGAGC TGACC AGCG CCGCCC TCCC CCGCCC CCGACC -3'
3'-TCCTCG ACTGG TCGC GGCGGG AGGG GGCGGG GGCTGG -5'
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Fig. 1. Sequence of the Half-ERE/Sp1 Binding Site

The sequence of the half-ERE/Sp1 binding site in the PR-A promoter originally reported by Kastner *et al.* (24) is shown. The locations of the ERE half-site and the proximal and distal Sp1 sites ($Sp1_P$ and $Sp1_D$, respectively) are indicated.

gous promoter, transient cotransfection experiments were carried out with a human ER expression vector and a chloramphenicol acetyltransferase (CAT) reporter plasmid containing either a TATA box alone (TATA CAT) or in combination with the half-ERE/Sp1 binding site (ERE/Sp1-TATA CAT). Exposure of transiently cotransfected CHO cells to E_2 resulted in a 1.7-fold increase in CAT activity when the reporter plasmid contained the half-ERE/Sp1 binding site (Fig. 3, ERE/Sp1-TATA CAT). This difference was statistically significant. In contrast, no statistical difference in activity was observed with E_2 treatment when the parental TATA CAT reporter plasmid was used. These findings suggest that the half-ERE/Sp1 binding site is involved in estrogen-mediated activation of the PR-A promoter.
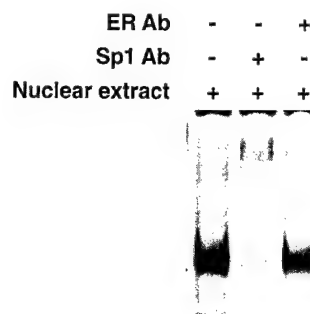
Proteins Present in MCF-7 Nuclear Extracts Bind to the Half-ERE/Sp1 Binding Site *in Vitro*

Our *in vivo* footprinting and transient transfection experiments provided evidence for the involvement of the half-ERE/Sp1 binding site in mediating estrogen's effects on the PR-A promoter. However, these studies did not allow us to identify proteins that interact with this DNA sequence. To begin to identify proteins that bind to this site, gel mobility shift assays were carried out with MCF-7 nuclear extracts. When ^{32}P-labeled oligos, each containing the half-ERE/Sp1 binding site, were combined with nuclear extracts prepared from E_2-treated MCF-7 cells, one major protein-DNA complex was formed (Fig. 4, lane 1). Since we anticipated that ER and Sp1 might bind to this region, antibodies to these proteins were included in separate binding reactions. The major protein-DNA complex was supershifted by the Sp1-specific antibody 1C6, which binds

1 2 3

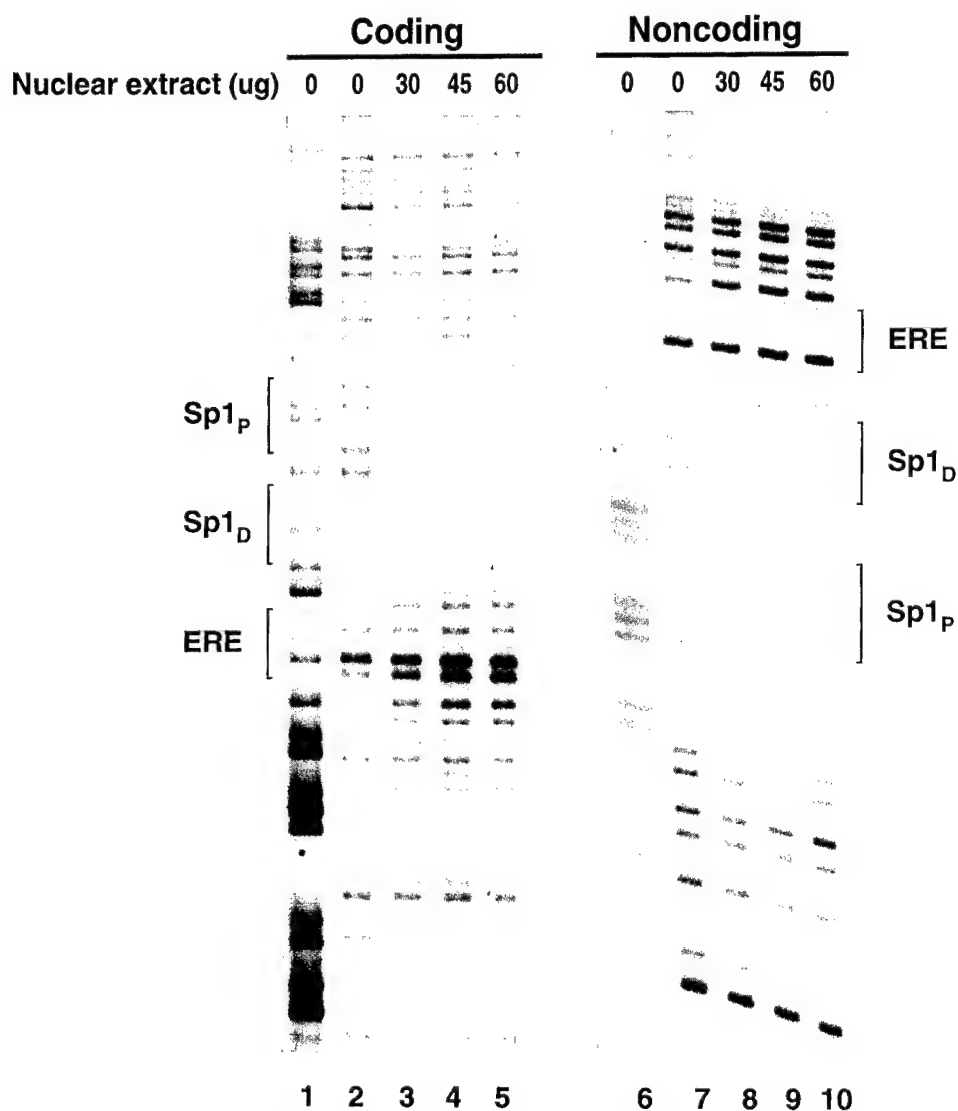**Fig. 4.** Binding of MCF-7 Sp1 Protein to the Half-ERE/Sp1 Binding Site

^{32}P-labeled oligos containing the half-ERE/Sp1 binding site were incubated with nuclear extracts from E_2-treated MCF-7 cells. The ER-specific antibody H222 (ER Ab) or the Sp1-specific antibody IC6 (Sp1 Ab) was added to the binding reaction as indicated. The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

to Sp1, but does not cross-react with Sp2–4 (lane 2), suggesting that Sp1 was present in substantial amounts in our MCF-7 nuclear extracts and that it bound efficiently to the half-ERE/Sp1 binding site. In contrast, the major protein-DNA complex was not affected by the ER-specific antibody H222 (lane 3).

Although our gel mobility shift experiments suggested that Sp1 was involved in regulation of the PR gene expression, they did not provide evidence that the ER was involved in formation of a protein-DNA complex. However, gel mobility shift experiments require the formation of stable protein-DNA complexes, which must be maintained during extended periods of electrophoresis. To determine whether a more transient or lower affinity interaction might occur between MCF-7 nuclear proteins and the ERE half-site and/or either one or both of the Sp1 binding sites, *in vitro* DNase I footprinting was carried out.

Fold Induction

Fig. 3. Estrogen-Enhanced Activity of a Plasmid Containing the Half-ERE/Sp1 Binding Site

CHO cells were transfected with TATA CAT or ERE/Sp1-TATA CAT reporter plasmid, hER expression plasmid, β-galactosidase expression plasmid, and pTZ nonspecific DNA using the calcium phosphate coprecipitation method as described in *Materials and Methods*. Cells were treated with ethanol vehicle or 10 nM E_2. Data represent the average of 9 independent experiments. Values are presented as the mean ± SEM. *, $P < 0.02$ for E_2 *vs.* ethanol control by Student's *t* test when ERE/Sp1-TATA CAT was used.

DNA fragments (181 bp), each containing the half-ERE/Sp1 binding site and additional PR flanking sequence, were [32]P-labeled on one end, incubated with increasing amounts of MCF-7 nuclear extract, and exposed to limited DNase I cleavage (Fig. 5, lanes 3–5 and 8–10). When DNA fragments that had been [32]P-labeled on the coding strand were used, the proximal and distal Sp1 sites were partially protected by proteins present in the MCF-7 nuclear extracts (lanes 3–5). Quantitative analysis of the coding strand revealed slightly greater protection of the proximal Sp1 site than the distal Sp1 site. Although the ERE half-site was not protected, nucleotides within and immediately flanking the ERE half-site were hypersensitive to DNase I cleavage upon addition of increasing concentrations of nuclear proteins (lanes 3–5). When the noncoding DNA strand was labeled and used in *in vitro* footprinting experiments with MCF-7 nuclear extracts, the proximal Sp1 site was more extensively protected than the distal Sp1 site (lanes 8–10). As seen with the coding strand, hypersensitive sites were observed within and adjacent to the ERE half-site on the noncoding strand. Control lanes containing DNA fragments that had been exposed to dimethylsulfate (DMS) (lanes 1 and 6) or DNase I (lanes 2 and 7) in the absence of protein were included for reference. The enhanced protection of the Sp1 sites observed in our *in vitro*

Fig. 5. *In Vitro* Footprinting of the Half-ERE/Sp1 Binding Site with MCF-7 Nuclear Extracts
DNA fragments (181 bp) containing the half-ERE/Sp1 binding site were end-labeled on either the coding or noncoding strand and incubated with increasing concentrations of nuclear extract from E$_2$-treated MCF-7 cells (lanes 3–5 and 8–10). The binding reactions were subjected to limited DNase I digestion, and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments, which had been treated *in vitro* with either DMS (lanes 1 and 6) or DNase I (lanes 2 and 7), were included as references. The locations of the proximal Sp1 site (Sp1$_P$), distal Sp1 site (Sp1$_D$), and ERE half-site are indicated.

footprints in the presence of MCF-7 nuclear extracts was similar to the increased protection of the Sp1 sites in the endogenous gene upon E_2 treatment of MCF-7 cells. The ERE half-site was not protected in our *in vitro* footprints as seen in the *in vivo* footprints, but rather displayed hypersensitivity to DNase I cleavage on both strands. Since DNase I hypersensitivity can result from binding of a protein to the major groove of the DNA helix, making the minor groove more accessible to DNase I cleavage (28), the hypersensitivity observed within and adjacent to the ERE could result from binding of a protein to the major groove in the region of the ERE.

Purified Sp1 Binds to the Half-ERE/Sp1 Binding Site

Our antibody supershift experiments indicated that native Sp1 present in MCF-7 nuclear extracts was binding to the half-ERE/Sp1 binding site. However, the MCF-7 extracts used in these assays contained a complex combination of nuclear proteins. To determine whether Sp1 protein alone was capable of binding to the half-ERE/Sp1 binding site or whether other proteins present in MCF-7 nuclear extracts were required for Sp1 binding, gel mobility shift experiments were carried out with purified Sp1 protein. [32]P-labeled oligos, each containing the half-ERE/Sp1 binding site, were incubated with increasing concentrations of purified Sp1 protein and fractionated on a nondenaturing acrylamide gel (Fig. 6, lanes 2–5). At the lowest Sp1 concentration used (1 ng), a single gel-shifted band was observed (←1, lane 2). As increasing concentrations of Sp1 were added to the binding reaction, there was a dose-dependent increase in a second, higher mol wt complex (←2, lanes 3–5). These experiments demonstrate that purified Sp1 was capable of forming a stable complex with the half-ERE/Sp1 binding site. Additional gel shift assays demonstrated that the more rapidly migrating Sp1-DNA complex had the same mobility as the complex formed with MCF-7 nuclear extracts (data not shown).

It seemed likely that the formation of the higher order complex in our gel shift experiments represented the simultaneous binding of two Sp1 proteins to the two Sp1 sites and the more rapidly migrating complex represented Sp1 binding to one of the two Sp1 sites. To determine whether Sp1 was binding to one or both of the Sp1 sites and whether it displayed any preference in binding to the proximal or the distal Sp1 site, *in vitro* footprinting experiments were carried out. DNA fragments (181 bp), each containing the half-ERE/Sp1 binding site, were [32]P-labeled on the coding strand and incubated with increasing concentrations of purified Sp1 protein. When 12.5 ng of purified Sp1 were included in the binding reaction, the proximal and distal Sp1 sites were protected (Fig. 7, lanes 3). Addition of 25 and 37.5 ng of purified Sp1 protein further enhanced protection of the two Sp1 sites (lanes 4 and 5). When


Fig. 6. Gel Mobility Shift Assay of half-ERE/Sp1 Binding Site-Containing Oligos and Purified Sp1 Protein
[32]P-labeled oligos containing the half-ERE/Sp1 binding site were incubated alone (lane 1) or with increasing concentrations of purified Sp1 protein (lanes 2–5) and fractionated on a nondenaturing gel. The locations of the more rapidly (←1) and more slowly (←2) migrating Sp1/DNA complexes are indicated.

DNA fragments labeled on the noncoding strand were incubated with increasing amounts of purified Sp1, the proximal Sp1 site was more protected than the distal Sp1 site (lanes 8–10). This preference for the proximal Sp1 site was also evident in the *in vitro* footprints of the noncoding strand in the presence of MCF-7 nuclear extracts (Fig. 5). Control lanes containing DNA fragments that had been exposed to DMS (Fig. 7, lanes 1 and 6) or DNase I (lanes 2 and 7) in the absence of proteins were included for reference. These data, combined with our gel mobility shift assays, support the idea that Sp1 binds first to the proximal Sp1 site and then to the distal Sp1 site.

ER Enhances Sp1 Binding to the Half-ERE/Sp1 Binding Site

Our *in vitro* binding assays suggested that Sp1 was involved in regulating the PR gene, but left some ques-
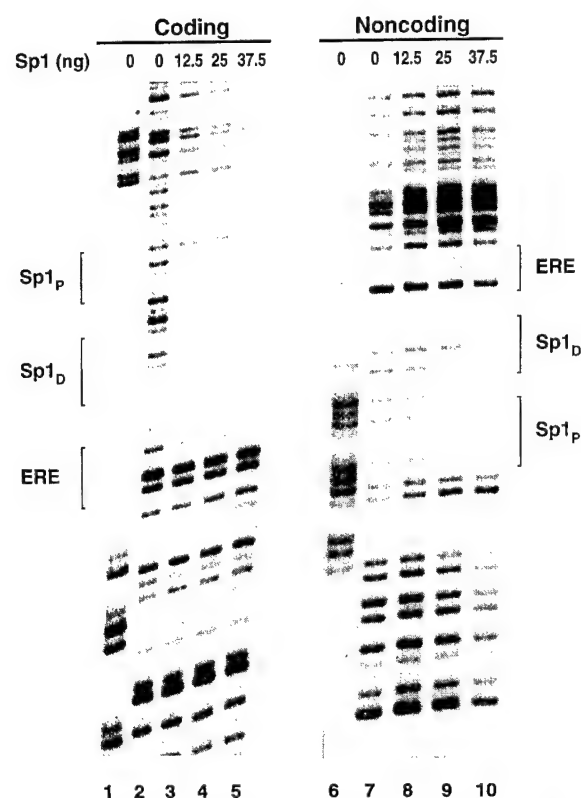
Fig. 7. *In Vitro* Footprinting of the Half-ERE/Sp1 Binding Site with Purified Sp1

DNA fragments (181 bp) containing the half-ERE/Sp1 binding site and flanking regions were end-labeled on either the coding or noncoding strand and incubated with increasing concentrations of purified Sp1 protein (lanes 3–5 and 8–10). The binding reactions were subjected to limited DNase I digestion, and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments that had been treated *in vitro* with either DMS (lanes 1 and 6) or DNase I (lanes 2 and 7) were included as references. The locations of the proximal Sp1 site (Sp1$_P$), distal Sp1 site (Sp1$_D$), and ERE half-site are indicated.

tion about the involvement of ER in this process. From previous studies examining ER-mediated transcription activation, it seemed possible that ER could increase transcription either directly by binding to the ERE half-site or indirectly by enhancing Sp1 binding (7–12, 29, 30). To determine whether ER affected protein-DNA complex formation, gel mobility shift assays were carried out. When a ^{32}P-labeled oligo containing the half-ERE/Sp1 binding site was incubated with 3 ng of purified Sp1, a single gel-shifted band was formed (Fig. 8A, lane 1, Sp1→). When the amount of purified Sp1 protein was decreased to 0.25 ng, a faint gel-shifted band was barely visible (lanes 2 and 8). Addition of 150, 350, or 700 fmol of purified, unoccupied (lanes 3–7), or E$_2$-occupied (lanes 9–13) receptor to 0.25 ng purified Sp1 protein elicited a dose-dependent increase in Sp1 binding. This ER-enhanced Sp1 binding was not due to an increase in protein concentration since all reactions contained the same amount of total

protein. Incremental addition of ER also resulted in a dose-dependent increase in a more rapidly migrating protein-DNA complex. The ER-specific antibody H151 supershifted this more rapidly migrating complex (ER→) but did not affect the Sp1-DNA complex (lanes 6 and 12). The Sp1-specific antibody IC6 supershifted the Sp1-DNA complex but did not affect the more rapidly migrating ER-DNA complex (lanes 7 and 13). These findings demonstrate that ER enhances Sp1 binding and that both ER and Sp1 can bind directly to the half-ERE/Sp1 binding site.

In addition to the major ER-DNA and Sp1-DNA complexes, three minor, higher order protein-DNA complexes, which we thought might reflect the formation of a trimeric ER-Sp1-DNA complex, were consistently observed in our gel shifts (Fig. 8A, lanes 5 and 11). To determine whether this was the case, ^{32}P-labeled oligos containing the half-ERE/Sp1 binding site were incubated with ER in the absence and in the presence of Sp1. When ER, but not Sp1, was included in the binding reaction (Fig. 8B, lanes 1–4), the major ER-DNA complex was formed (←ER) as well as two minor, higher order complexes, which most likely contained ER and Sf-9 proteins that co-purified with the receptor. When 1 ng Sp1 and 350 or 700 fmol ER were included in the binding reaction (lanes 6–9), the major ER (←ER) and Sp1 (←Sp1) complexes and three minor, higher order complexes were formed. The one unique, higher order protein-DNA complex (lanes 6 and 7,←ER/Sp1) formed in the presence of both ER and Sp1 was supershifted by ER- and Sp1-specific antibodies, demonstrating that both ER and Sp1 were present. A lane containing Sp1 alone was included as a reference (lane 5). These combined experiments suggest that ER and Sp1 can form a trimeric complex with DNA at the half-ERE/Sp1 binding site in the PR-A promoter.

To determine how ER affected Sp1 protection of the half-ERE/Sp1 binding site, *in vitro* DNase I footprinting experiments were carried out with purified ER and Sp1 proteins. When 15 ng of purified Sp1 were incubated with the ^{32}P-labeled coding strand, the proximal and distal Sp1 sites were protected (Fig. 9, lane 3). Addition of 15 ng Sp1 and 0.33–1.3 pmol of purified ER incrementally enhanced the protection of both the proximal and distal Sp1 sites (lanes 4–6). As suggested from the gel mobility shift assays, the consensus ERE half-site was protected in the presence of higher ER concentrations (lane 6). When DNA fragments labeled with ^{32}P on the noncoding strand were incubated with 15 ng of purified Sp1 and increasing concentrations of purified ER, enhanced protection of both the proximal and distal Sp1 sites and the half-ERE was observed (lanes 9–12). As seen in the *in vitro* footprints with MCF-7 nuclear extracts and with purified Sp1, the proximal Sp1 site on the noncoding strand was more extensively protected than the distal Sp1 site. The ERE half-site was partially protected on the noncoding strand. Control lanes containing DNA fragments that had been exposed to DMS (lanes 1 and
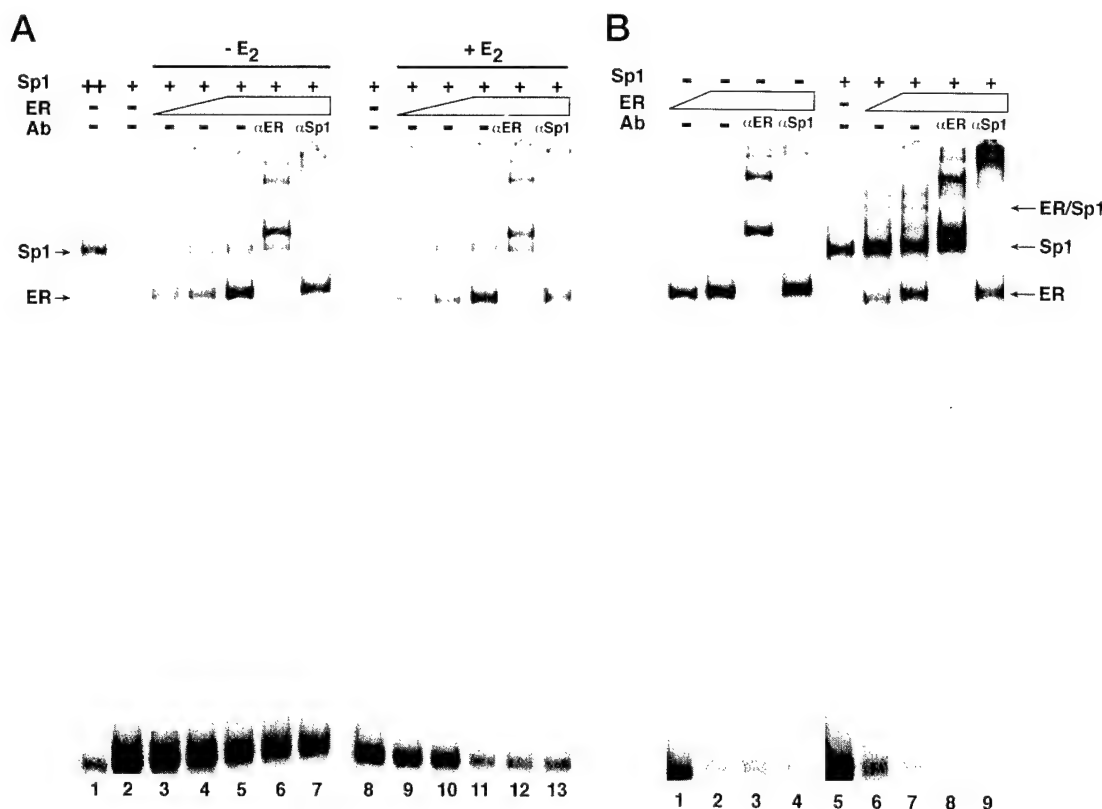
Fig. 8. ER-Enhanced Binding of Sp1 to the Half-ERE/Sp1 Binding Site

[32]P-labeled oligos containing the half-ERE/Sp1 binding site were incubated with purified Sp1, ER, or Sp1 and ER and fractionated on nondenaturing gels. A, Binding reactions contained either 3 ng of Sp1 (lane 1) or 0.25 ng of Sp1 alone (lanes 2 and 8) or in combination with 150 (lanes 3 and 9), 350 (lanes 4 and 10), or 700 (lanes 5–7 and 11–13) fmol of unoccupied ($-E_2$) or E_2-occupied ($+E_2$) ER. B, Binding reactions contained 350 (lane 1) or 700 (lanes 2–4) fmol of ER, 3 ng of Sp1 (lane 5), or 1 ng of Sp1 (lanes 6–9) and 350 (lane 6) or 700 (lanes 7–9) fmol of ER. The ER-specific antibody H151 (ER Ab) or the Sp1-specific antibody IC6 (Sp1 Ab) was added as indicated. The locations of the ER/DNA ($\leftarrow$ER), Sp1/DNA ($\leftarrow$Sp1), and ER/Sp1/DNA ($\leftarrow$ER/Sp1) complexes are indicated.

7) or DNase I (lanes 2 and 8) in the absence of proteins were included for reference.

Sp1 Does Not Enhance ER Binding to the ERE Half-Site

Our binding assays indicated that ER greatly enhanced binding of Sp1 to the half-ERE/Sp1 binding site. To determine whether Sp1 influenced ER binding, gel mobility shift assays were carried out. When [32]P-labeled oligos containing the half-ERE/Sp1 binding site were incubated with 150 fmol of purified ER, a single major gel shifted band was formed (Fig. 10, lane 1, $\leftarrow$ER). When the amount of purified ER protein was decreased to 50 fmol, a fainter gel shifted band was produced (lanes 2–6, $\leftarrow$ER). Addition of 0.25, 0.5, 1.5, or 3 ng of purified Sp1 protein (lanes 3–6, $\leftarrow$Sp1) elicited a dose-dependent increase in Sp1 binding. In contrast, incremental addition of Sp1 slightly decreased ER binding to the half-ERE/Sp1 binding site. These combined findings demonstrate that although ER greatly enhanced Sp1 binding, Sp1 did not enhance ER binding to the half-ERE/Sp1 binding site.

Purified Sp1 and ER Bind Differentially to Wild-Type and Mutant Half-ERE/Sp1 Binding Sites

The *in vitro* footprinting experiments reproducibly suggested a preference of Sp1 for the proximal Sp1 site. To determine how each of the Sp1 sites and the ERE half-site contributed to protein-DNA complex formation, each of the individual elements was mutated and tested in gel mobility shift assays. Complementary oligos containing the wild-type half-ERE/Sp1 binding site (wt) or mutations in both Sp1 sites (mP/D), the distal Sp1 site (mD), the proximal Sp1 site (mP), or the ERE half-site (mE) were synthesized, annealed, and labeled with [32]P. The labeled oligos were combined with purified Sp1 (Fig. 11, lanes 1–5) or purified Sp1 and ER (lanes 6–10) and fractionated on nondenaturing gels. Sp1 or Sp1 and ER bound effectively to the wt half-ERE/Sp1 binding site (lanes 1 and 6). As anticipated, Sp1 did not bind to the oligo containing mutations in both Sp1 sites, in the absence (lane 2) or in the presence of ER (lane 7). Sp1 alone or in combination with ER bound to oligos containing a mutation in one
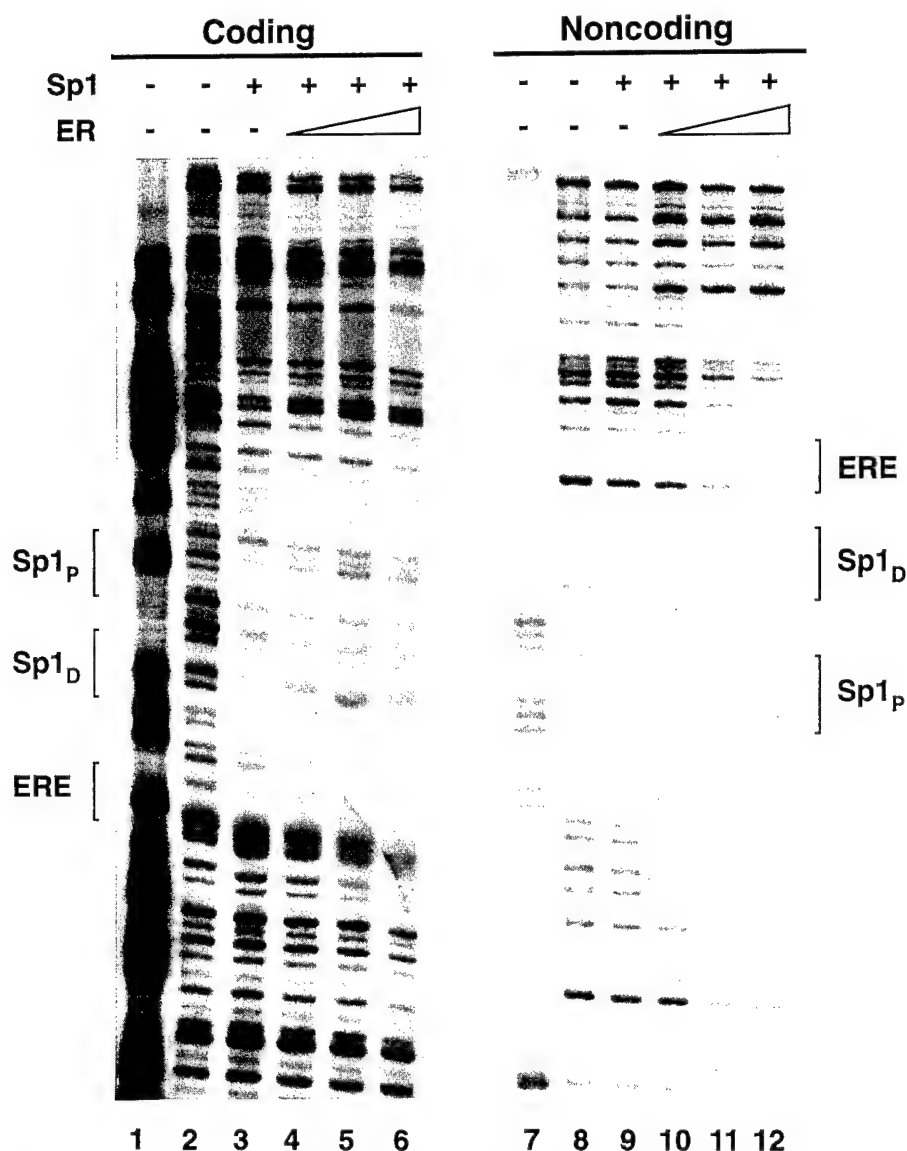
Fig. 9. *In Vitro* DNase I Footprinting of the Half-ERE/Sp1 Binding Site with Purified Sp1 and ER
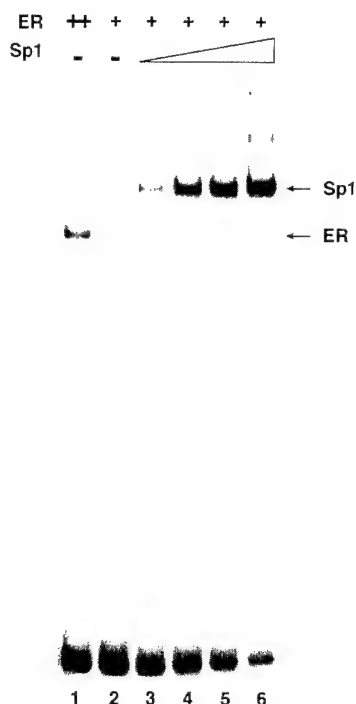
DNA fragments containing the half-ERE/Sp1 binding site and flanking regions were end-labeled on either the coding or noncoding strand and incubated with 15 ng of purified Sp1 protein (lanes 3–6 and 9–12) and 0.33, 0.66, or 1.3 pmol of purified ER (lanes 4–6 and 10–12). The binding reactions were subjected to limited DNase I digestion, and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments that had been treated *in vitro* with either DMS (lanes 1 and 7) or DNase I (lanes 2 and 8) were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$), and ERE half-site are indicated.

of the two Sp1 sites, but more protein-DNA complex was formed when the oligo contained an intact proximal Sp1 site (mD; compare lanes 3 and 8 with lanes 4 and 9). PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) analysis of four gel shift experiments demonstrated that there was a 30.8% ($\pm$7 SEM) increase in complex formation with the proximal Sp1 site compared with the distal Sp1 site. These findings corroborate the preferential binding of Sp1 to the proximal Sp1 site observed in the *in vitro* footprinting studies. The ability of ER to bind to oligos containing an intact ERE half-site (lanes 6–9), but not to an oligo

containing a mutated ERE half-site (lane 10), further supports the idea that ER can bind to the ERE half-site. When the ERE half-site was mutated, increased Sp1-DNA complex formation was observed (lanes 5 and 10). The reason for this apparent increase in Sp1 binding is unclear, but it was a reproducible finding.
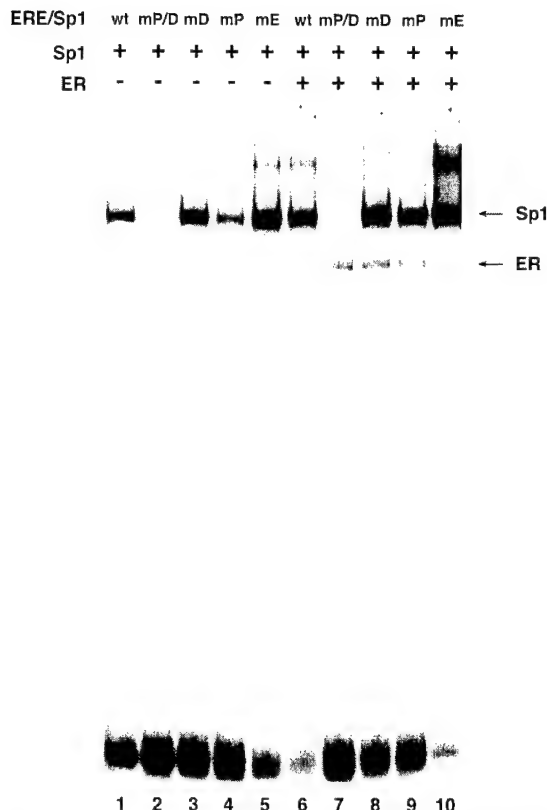
DISCUSSION

Sequence comparison of the PR gene from different species has been used to identify cis elements that are

Fig. 10. Effect of Sp1 on ER Binding to the Half-ERE/Sp1 Binding Site

[32]P-labeled oligos containing the half-ERE/Sp1 binding site were incubated with 150 fmol (lane 1) or 50 fmol of purified ER (lanes 2–6) and 0.25, 0.5, 1.5, or 3 ng of purified Sp1 (lanes 3–6) and fractionated on nondenaturing gels. The locations of the ER-DNA (←ER) and Sp1-DNA (←Sp1) complexes are indicated.

Fig. 11. Interaction of Purified Sp1 and ER with wt and Mutant Half-ERE/Sp1 Binding Sites

[32]P-labeled oligos containing the wild-type half-ERE/Sp1 binding site (wt), or mutations in both Sp1 binding sites (mP/D), the distal Sp1 binding site (mD), the proximal Sp1 binding site (mP), or the ERE half-site (mE) were incubated with 3 ng of purified Sp1 (lanes 1–5) or 3 ng of purified Sp1 and 0.33 pmol of purified ER (lanes 6–10). The [32]P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography. The locations of the ER-DNA (←ER) and Sp1-DNA (←Sp1) complexes are indicated.

involved in estrogen-regulated transactivation. The rabbit PR gene contains an imperfect ERE, which overlaps with the translation start site and is capable of conferring estrogen responsiveness to a heterologous promoter in transient transfection assays (31). Although a similar sequence is present in the chicken PR gene (32), no homologous sequence has been identified in the human PR gene (24). A number of studies have suggested that ER and Sp1 may be involved in conferring estrogen responsiveness to the creatine kinase B (7), c-*myc* (8), retinoic acid receptor α (9), heat shock protein 27 (10, 11), cathepsin D (12), and uteroglobin (13) genes. The identification of an ERE half-site adjacent to two Sp1 sites in the human PR gene (24) led us to investigate whether this region might be involved in conferring estrogen-responsiveness to the human PR gene. We initiated our studies by examining the endogenous PR gene in MCF-7 cells. Unlike transient transfection assays, which examine the ability of ER to activate transcription of synthetic promoters in supercoiled plasmids, our *in vivo* DNase I footprinting experiments allowed us to examine the endogenous PR gene as it exists in native chromatin and assess whether the half-ERE/Sp1 binding site might play a physiological role in gene expression. E_2 treatment of MCF-7 cells did elicit more ex-

tensive protection of the half-ERE/Sp1 binding site than was observed in the absence of hormone. The enhanced protection of the half-ERE/Sp1 binding site seen after 72 h of hormone treatment, a time when PR mRNA and protein reach maximal levels (16–18, 26, 27), suggests that sustained protein-DNA interactions are required for maximal production of PR mRNA and protein. Furthermore, the ability of the half-ERE/Sp1 binding site to enhance transcription of a CAT reporter plasmid in the presence of E_2 suggests that this region is involved in estrogen responsiveness of the PR-A promoter.

A Role for Sp1 in Regulating Expression of the PR Gene

Sp1 was originally described as a *trans*-acting factor that bound to a GC box (5'-GGGCGG-3') and activated transcription of the SV40 promoter (33, 34). Subsequent comparison of numerous Sp1 binding sites

led to the identification of a higher affinity, consensus Sp1 site, 5'-GGGGCGGGGC-3' (35), and the discovery that sequences that varied from this consensus sequence displayed decreased affinities for Sp1. While both of the Sp1 sites in the human PR half-ERE/Sp1 binding site contain the GC box motif, only the proximal Sp1 site contains the 10-bp consensus Sp1 sequence (Fig. 1). The increased affinity of Sp1 for the 10-bp proximal Sp1 site, when compared with the distal Sp1 site, was repeatedly observed in our *in vitro* footprinting assays and was most obvious on the noncoding strand (Figs. 5, 7, and 9). Gel mobility shift assays carried out with oligos containing mutations in the proximal or distal Sp1 site confirmed Sp1's preference for the proximal Sp1 site (Fig. 11). Interestingly, the centers of the two GC boxes present in the half-ERE/Sp1 binding site are separated by 10 bp or one turn of the DNA helix ($Sp1_D$ +580 to +585, $Sp1_P$ +590 to +595). The periodicity of these elements could either favor interaction between adjacent DNA-bound proteins resulting in cooperative binding or sterically hinder binding of two Sp1 proteins. Our gel mobility shift and *in vitro* DNase I footprinting assays provided evidence for additive, not cooperative, binding of Sp1 to these sites and indicate that Sp1 binds first to the proximal Sp1 site and then to the distal Sp1 site.

A Role for ER in Regulating Expression of the PR Gene

One way that estrogen might affect PR gene expression is through direct binding of the receptor to the ERE half-site. The ERE was protected in our *in vitro* footprinting experiments with ER and Sp1, but not with Sp1 alone, demonstrating that the ER did bind to the ERE half-site. Likewise, gel mobility shift experiments carried out with purified ER alone or in combination with Sp1 indicated that the ER bound surprisingly well to the ERE half-site and formed a stable protein-DNA complex that was capable of withstanding the extensive periods of electrophoresis required for gel mobility shift experiments. Furthermore, the ERE half-site was protected in our *in vivo* footprinting experiments after treatment of MCF-7 cells with E_2, suggesting that this element is involved in regulation of the endogenous gene. Although we were unable to detect protection of the ERE half-site in our *in vitro* binding assays using MCF-7 nuclear extracts, the level of ER in these extracts (0.42 fmol/μg protein) was significantly lower than the level present in an intact cell nucleus. Assuming a nuclear radius of 6 μm and 150,000 ER sites per cell (36), the ER concentration in an MCF-7 nucleus would be 273 nM. These ER concentrations are significantly higher than the 7–57 nM concentrations used in our *in vitro* binding assays and would most likely favor ER binding to the ERE half-site. The 10 bp separating the ERE half-site and the distal Sp1 binding site would place the ER on the same side of the DNA helix as the DNA-bound Sp1 proteins and could help to foster protein-protein interactions.

Estrogen could also modulate PR gene expression through ER-enhanced Sp1 binding. ER effectively enhanced Sp1 binding to the two Sp1 sites in the PR-A promoter and formed a trimeric complex with Sp1 and DNA in our *in vitro* binding assays. Direct ER-Sp1 interaction has also been documented in immunoprecipitation and glutathione-S-transferase (GST) pulldown experiments (11, 29).

We have considered only ERα in our studies since MCF-7 cells express high levels of ERα, but do not express ERβ (36, 37). Although we have not ruled out the possibility that another nuclear protein might bind to the ERE half-site, the high levels of nuclear ER, the differential protection of the ERE half-site in the presence and absence of E_2, and the demonstrated ability of ER to bind to the ERE half-site *in vitro* suggest that it is most likely the ER that interacts with this site *in vivo* and helps to regulate transcription of the PR-A promoter.

Regulation of the PR-A Promoter in MCF-7 Cells

Estrogen treatment of transfected cells resulted in a modest, but reproducible 1.7-fold increase in transcription of a plasmid containing the ERE/Sp1 binding site. Since estrogen treatment of MCF-7 cells results in a 2- to 10-fold increase in PR mRNA levels (16–18), it seems likely that the ERE/Sp1 binding site may play a significant role in mediating the estrogen responsiveness of this gene. However, the ERE/Sp1 site represents a small part of the complex PR promoter, which contains multiple regulatory elements. Unlike promoters that contain a palindromic ERE, transcription of the estrogen-regulated PR gene may require ER action at multiple *cis* elements. Preliminary experiments from our laboratory suggest that additional Sp1 and AP1 sites in the PR promoter may also be involved in estrogen-regulated gene expression (L. Petz and A. Nardulli, unpublished data). Thus, the cooperative action of the multiple sites within the PR promoter may be required for effective estrogen-regulated transcription.

Our studies support the idea that ER and Sp1 are involved in estrogen-regulated expression of the human PR-A promoter. The protection of nucleotides flanking the half-ERE/Sp1 binding site in our *in vivo* footprinting experiment suggests that other proteins are associated with the promoter and are involved in transcription activation. Interestingly, the E_2-occupied ER, but not the unoccupied ER, interacts with a number of coactivator proteins, which participate in transcription activation and chromatin remodeling (38–46). The recruitment of these proteins to the DNA-bound, liganded receptor could account for protection of sequences flanking the half-ERE/Sp1 binding site and serve as the initiating event in the formation of an active transcription complex.

While models of DNA are typically drawn in a linear array, the packaging of DNA and protein into the nucleus of a cell requires tremendous compaction. This

compaction could facilitate interaction between *trans* acting factors bound to more distant *cis* elements. Thus, the association of upstream activators, such as ER and Sp1, with factors bound to downstream elements could be fostered. In fact, both ER and Sp1 are known to directly associate with TFIID components. ER interacts with TATA binding protein (TBP), transcription factor IIB (TFIIB), and TBP-associated factor $(TAF)_{II}30$ (47–49), and Sp1 interacts with TBP, $TAF_{II}130$, and $TAF_{II}55$ (50–53). The interaction of ER and Sp1 with TBP and its associated proteins could foster formation of a protein-protein network that helps to establish an active transcription complex. Furthermore, the E_2-dependent recruitment of coactivators such as CBP/p300, which can function as a histone acetyltransferase (39), could help remodel chromatin in the PR-A promoter and enhance formation of an interconnected protein-protein and protein-DNA network involved in activation of the human PR gene.

MATERIALS AND METHODS

Cell Culture

MCF-7 human breast cancer cells (54) were maintained in Eagle's MEM containing 5% heat-inactivated calf serum. Cells were seeded in 10-cm plates and transferred to phenol red-free, serum-free improved MEM (55) 5 days before the experiments were conducted. Chinese Hamster Ovary (CHO) cells were maintained in DMEM/F12 supplemented with 5% charcoal dextran-stripped calf serum (27).

Oligonucleotides and Plasmid Constructions

The names and sequences of wild-type (wt) or mutant half-ERE/Sp1 binding sites are listed. Nucleotides that differ from the endogenous, wt half-ERE/Sp1 binding site are *underlined*.

ERE/Sp1 wt: 5'-GATCTAGGAGCTGACCAGCGCCGCCC-TCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGGGCGGCGC-TGGTCAGCTCCTA-3',

ERE/Sp1 mP/D: 5'-GATCTAGGAGCTGACCAGCGTTG-TACTCCCTTGTACCCGACCA-3'

and 5'-GATCTGGTCGGGTACAAGGGAGTACAACGCT-GGTCAGCTCCTA-3',

ERE/Sp1 mD: 5'-GATCTAGGAGCTGACCAGCGTTGTA-CTCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGTACAACGCT-GGTCAGCTCCTA-3',

ERE/Sp1 mP: 5'-GATCTAGGAGCTGACCAGCGCCGC-CCTCCCTTGTACCCGACCA-3'

and 5'-GATCTGGTCGGGTACAAGGGAGGGCGGCGCT-GGTCAGCTCCTA-3',

ERE/Sp1 mE: 5'-GATCTAGGAGCTGATTAGCGCCGCCC-TCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGGGCGGCGC-TAATCAGCTCCTA-3'.

ERE/Sp1 wt oligos with *Bgl*II compatible ends were subcloned into the *Bgl*II-cut, dephosphorylated CAT reporter plasmid, TATA CAT (56), to create ERE/Sp1-TATA CAT. The ligated vector was transformed into the DH5α strain of *Escherichia coli*, sequenced, and purified on two cesium chloride gradients.

In Vitro and *in Vivo* Treatment of Genomic DNA

MCF-7 cells were exposed to ethanol vehicle or 1 nM E_2 for 0, 2, or 72 h before DNase I treatment. Cells were permeabilized with 0.4% NP-40 and treated with 750 U DNase I/ml (Roche Molecular Biochemicals, Indianapolis, IN) for 3 min at 25 C. Isolation of genomic DNA was carried out as described by Mueller and Wold (25). The genomic DNA was purified, incubated with RNase A, resuspended in TE (10 mM Tris, pH 7.5, 1 mM EDTA) and stored at −20 C.

Naked genomic DNA was treated *in vitro* with DMS as described (25). *In vitro* DNase I-treated DNA was prepared by adjusting 100 µg of protein-free, RNase A-treated DNA to 175 µl with TE. DNA was incubated with 2.5×10^{-5} U DNase I for 5 min at 37 C. The reaction was stopped by the addition of 10 mM EDTA and processed as described for *in vivo*-treated genomic DNA.

In Vivo Footprinting

Ligation-mediated PCR (LMPCR) footprinting was carried out essentially as described by Mueller and Wold (25, 57). Two micrograms of genomic DNA were subjected to LMPCR procedures using nested primers, which annealed to sequences upstream of the half-ERE/Sp1 binding site (+571 to +595) in the human PR gene. The primer sequences were: primer 1, 5'-TCCCCGAGTTAGGAGACGAGAT-3'; primer 2, 5'-CGCTC-CCCACTTGCCGCTC-3'; and primer 3, 5'-GCTCCCCACTT-GCCGCTCGCTG-3'. The annealing temperatures for the primers were 55 C, 62 C, and 69 C, respectively. The linker primers LMPCR 1 and LMPCR 2 described by Mueller and Wold (57) were also used, except that LMPCR 1 was modified by removing the two 5'-nucleotides to eliminate potential secondary structure.

In Vitro DNase I Footprinting

Primers, which annealed 88 bp upstream (primer 3) or 79 bp downstream (primer 4, 5'-TCGGGAATATAGGGGCAGAGG-GAGGAGAA-3') of the half-ERE/Sp1 binding site, were subjected to 30 rounds of PCR amplification with 30 ng of the PR-(+464/+1105) CAT (24). Labeling of the coding and noncoding strands was carried out with ^{32}P-labeled primer 3 or primer 4, respectively. The 181-bp singly end-labeled amplified fragments were fractionated on an acrylamide gel and isolated. End-labeled DNA fragments (100,000 cpm) containing the half-ERE/Sp1 binding site were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM $MgCl_2$, 50 ng of poly dIdC, and 0.4 mM dithiothreitol (DTT) in a final volume of 50 µl with either 30–60 µg of MCF-7 nuclear extract, 12.5–37.5 ng of purified Sp1 protein (Promega Corp., Madison, WI), or 15 ng of purified Sp1 and 0.13–1.3 pmol of purified Flag-tagged ER, which had been expressed and purified as described by Kraus and Kadonaga (58). E_2 (10 nM) was included in binding reactions containing the purified ER. BSA, ovalbumin, and KCl were added as needed to maintain constant protein and salt concentrations. When MCF-7 nuclear extracts were used, poly dI/dC was increased to 1 µg per reaction. RQ1 ribonuclease-free DNase I (1–2 U) (Promega Corp., Madison, WI) was added to each sample and incubated at room temperature for 0.75–8 min. The DNase I digestion was terminated by addition of stop solution (200 mM NaCl, 1% SDS, 30 mM EDTA, and 100 ng/µl tRNA). The DNA was phenol/chloroform extracted, precipitated, and resuspended in formamide loading buffer (59). Samples were fractionated on an 8% denaturing acrylamide gel. Radioactive bands were visualized by autoradiography and quantitated with a PhosphorImager and ImageQuant software (Molecular Dynamics, Inc.).

Gel Mobility Shift Assays

Gel mobility shift assays were carried out essentially as described previously (60, 61). ^{32}P-labeled (10,000 cpm) half-ERE/Sp1-containing wt or mutant oligos were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM MgCl$_2$, 50 ng of poly dI/dC, and 0.4 mM DTT in a final volume of 20 μl with either 20 μg of MCF-7 nuclear extract, 0.25–3 ng of purified Sp1 protein, or 0.25 ng of purified Sp1 and 50–700 fmol of purified ER. E$_2$ (10 nM) was included in all binding reactions containing ER unless otherwise indicated. BSA, ovalbumin, and KCl were added as needed to maintain constant protein and salt concentrations. When MCF-7 nuclear extracts were used, the nonspecific DNA for each reaction included 1 μg of salmon sperm DNA, and poly dI/dC was increased to 2 μg. For antibody supershift experiments, the Sp1-specific monoclonal antibody, 1C6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or the ER-specific monoclonal antibody H222 or H151 (kindly provided by Drs. Geoffrey Greene, The University of Chicago, Chicago, IL and Dean Edwards, University of Colorado Health Science Center, Denver, CO, respectively) was added to the protein-DNA mixture and incubated for 10 min at room temperature. Low ionic strength gels and buffers were prepared as described previously (59). Radioactive bands were visualized by autoradiography and quantitated with a PhosphorImager and ImageQuant software (Molecular Dynamics, Inc.).

Transient Transfections

CHO cell transfections were performed using the calcium phosphate method (62). Crystals were formed in the presence of 3 μg of the indicated CAT reporter, 200 ng of the β-galactosidase vector pCH110 (Pharmacia Biotech, Piscataway, NJ), 5 ng of the human ERα expression vector pCMVhER (63), and 4.8 μg of pTZ18U and incubated with CHO cells for 16 h followed by a 2 min 20% glycerol shock. Cells were maintained in media containing ethanol vehicle or 10 nM E$_2$ for 24 h. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc. Hercules, CA) with BSA as a standard. Mixed-phase CAT assays were performed using 35 μg protein as previously described (64). The β-galactosidase activity was determined at room temperature as previously described (65) and used to normalize the amount of CAT activity in each sample.

Acknowledgments

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AP-1 Sites Are Involved in Conferring Estrogen Responsiveness to the Human Progesterone Receptor Gene

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ABSTRACT

The progesterone receptor (PR) gene is activated by estrogen in MCF-7 human breast cancer cells. Although the PR gene does not contain an estrogen response element (ERE), we identified two putative activating protein-1 (AP-1) sites at +90 and +745 in the PR gene that may play a role in conferring estrogen responsiveness. The +90 AP-1 site was hypersensitive to DNase I cleavage in genomic Southern analysis. The location and nucleotide sequence of the +745 AP-1 site are completely conserved in mouse, rat, human, and rabbit PR genes. Both AP-1 sites bound purified Fos and Jun, formed complexes with Fos-Jun heterodimers present in MCF-7 nuclear extracts, and functioned as estrogen-responsive enhancers in transient cotransfection assays. Estrogen treatment of MCF-7 cells transiently increased Fos levels and elicited a decrease in phosphorylated Jun and a concomitant increase in the dephosphorylated Jun. Chromatin immunoprecipitation assays demonstrated that the estrogen receptor was present at the +90 and +745 AP-1 sites in the endogenous PR gene after treatment of MCF-7 cells with estrogen. These studies suggest that the ability of estrogen to modulate Fos-Jun binding and the association of the estrogen receptor with the +90 and +745 AP-1 sites help confer estrogen responsiveness to the PR gene.

INTRODUCTION

17β-estradiol (E_2) is a hormone of crucial importance in the development and maintenance of reproductive tissues (1, 2) and also plays a critical role in cardiovascular, neural, and bone physiology (3-6). Estrogen's effects are mediated through its interaction with the intracellular estrogen receptor (ER). Classical models of estrogen action have considered the interaction of ERs α and β with estrogen response elements (EREs) as the initiating event involved in estrogen-regulated gene expression (7, 8). However, it has become apparent that, in addition to mediating its effects through the ERE, the ER can interact with other DNA-bound transcription factors to influence transcription activation. For example, the ER cooperates with the activating protein-1 (AP-1) proteins Fos and Jun to confer E_2 responsiveness to simple, heterologous promoters (9-11) and to the ovalbumin (12), c-*fos* (13), collagenase (9), and insulin-like growth factor I (14) genes. Likewise, the ER mediates estrogen's effects through Sp1 recognition sites in a number a genes including the progesterone receptor (PR) gene (15-21).

Previous studies have demonstrated that transcription of the PR gene is induced by E_2 in MCF-7 human breast cancer cells. PR mRNA and protein levels increase 2 to 10-fold reaching maximal levels after 72 hours of E_2 treatment (22-24). Like ER, two distinct PR forms, the 120 kD PR-B and the 94 kD PR-A, are expressed in a tissue-specific manner (25-27). Kastner et al (28) have hypothesized that two discrete promoters, A and B, are responsible for the production of PR-A and PR-B, respectively. In spite of the fact that both Promoter A (+464 to +1105) and Promoter B (-711 to +31) confer estrogen responsiveness to a heterologous promoter (28, 29), neither promoter contains an ERE.

We previously demonstrated that an ERE half site and two adjacent Sp1 sites in the human PR gene were involved in E_2-mediated activation of the PR gene (15). However, the

ability of this half ERE Sp1 site to mediate transcription activation was modest suggesting that other regions of the PR gene must also be involved in mediating estrogen's effects. We have identified two putative AP-1 sites in the PR gene that bound Fos and Jun *in vitro* and functioned as estrogen-inducible enhancers in transient transfection assays. ER was present at the +90 and +745 AP-1 sites in the endogenous PR gene only after MCF-7 cells were treated with E_2.

RESULTS

Identification of putative AP-1 sites in the human PR 5'-flanking region. To identify potential sites involved in regulation of the human PR gene, MCF-7 cells were treated with ethanol vehicle or E_2, nuclei were isolated and treated with increasing concentrations of DNase I, DNA was isolated, and Southern blot analysis was performed. When lower concentrations of DNase I (80-120 U/ml) were utilized, an 800 basepair band was observed (Fig 1, Lanes 3,4, 10, and 11). At higher DNase I (120 –240 U/ml) concentrations (Lanes 4-6 and 11-13), a 700 basepair band was present. It is interesting to note that both bands were present in the presence and in the absence of E_2. However, the bands were more pronounced in the presence of E_2 suggesting that these regions were somewhat accessible to DNase I cleavage in the absence of hormone and became more accessible to DNase I cleavage in the presence of E_2. The 700 and 800 basepair hypersensitive sites mapped to +1 and +90 of the PR gene (28), respectively. Examination of the +1 region failed to identify any putative regulatory element. Analysis of nucleotide sequence at the +90 hypersensitive site identified a putative AP-1 site (TGAGT<u>G</u>A) from +90 to +96, hereafter referred to as the +90 AP-1 site, that differs from a consensus AP-1 site (TGAG/CTCA, Ref. 30, 31) by one nucleotide. Nuclei that had not been exposed to DNase I (Lanes 1 and 8) produced a prominent 1.5 kb band, which resulted from *Bam* HI cleavage of the genomic DNA. Another

4

minor, lower molecular weight band, which resulted from a cross-reacting sequence (Data not shown), was sometimes visible.

An imperfect ERE, which overlaps with the translation start site of PR-B, was previously implicated in imparting estrogen-regulated gene expression to the rabbit PR gene (32). Examination of the corresponding region of the human PR gene indicated that this ERE was not conserved (28). However, we did identify a putative AP-1 site (TGACT$\underline{G}$A) in the region from +745 to +751, hereafter referred to as the +745 AP-1 site, that differed from a consensus AP-1 site (30, 31) by one basepair. This sequence is completely conserved in human, rat, mouse, and rabbit PR genes (Fig. 2).

$\underline{E_2}$ enhances transcription of reporter plasmids containing the +90 and +745 AP-1 sites. To determine whether either of the putative AP-1 sites could confer E_2-responsiveness, chloramphenicol acetyl transferase (CAT) reporter plasmids containing a TATA sequence alone (TATA-CAT) or in combination with either the +90 AP-1 site (+90 AP-1 TATA-CAT) or the +754 AP-1 site (+745 AP-1 TATA-CAT) were tested for their abilities to function as transcriptional enhancers. Cells were transfected with a CAT reporter plasmid and a β-galactosidase expression vector. A human ER expression vector was included as indicated (+ER). When the ER expression vector was used, exposure of transfected cells to E_2 resulted in 2.5- and 2.7-fold increases in CAT activity when the reporter plasmids contained the +90 and +745 AP-1 sites, respectively (Fig. 3). Although CAT activity was significantly different in vehicle- and E_2-treated cells when the reporter plasmids contained the +90 AP-1 site or the +745 AP-1 site, hormone treatment did not affect CAT activity when the reporter plasmid contained the TATA sequence alone. The estrogen-induced increase in CAT activity was dependent on the presence of ER, since no differences in CAT activity were observed when the ER expression vector was not

5

included (-ER). These findings demonstrate that the +90 AP-1 site and the +745 AP-1 site confer E_2-responsiveness to a heterologous promoter and may assist in mediating estrogen's effects on the endogenous PR gene. Purified Fos and Jun bind to oligos containing the +90 and +745 AP-1 sites. To determine whether Fos and Jun could bind to the +90 and +745 AP-1 sites, gel mobility shift experiments were carried out with purified Fos and Jun. ^{32}P-labeled oligos containing either the +90 (Fig. 4, Panel A) or the +745 (Panel B) AP-1 site were incubated with increasing concentrations of purified Fos and Jun and fractionated on a nondenaturing acrylamide gel. At the lowest Fos and Jun concentration utilized (0.005 μM), a faint gel-shifted band was observed (Lanes 2). As increasing concentrations of Fos and Jun were added to the binding reaction (Lanes 3-6), the intensity of the gel-shifted band increased. The protein-DNA complex was diminished by inclusion of either the Fos-specific antibody sc-52 (Lanes 7) or the Jun-specific antibody sc-45 (Lanes 8). Although equal concentrations of purified Fos and Jun were used, more protein-DNA complex was formed with the +745 site than the +90 AP-1 site. This enhanced binding to the +745 AP-1 site was especially evident when the lowest amounts of Fos and Jun were utilized (Lanes 2) and suggested that the +745 AP-1 site had a higher affinity for Fos and Jun than the +90 AP-1 site. These findings demonstrate that purified Fos and Jun were capable of forming a stable heterodimeric complex with both the +90 and +745 AP-1 sites. Parallel experiments performed with Fos or Jun alone indicated that neither Fos nor Jun homodimers bound to either AP-1 site (Data not shown).

Proteins present in E_2-treated MCF-7 nuclear extracts bind to the AP-1 sites. Our gel mobility shift assays indicated that purified Fos and Jun could bind to the +90 and the +745 AP-1 sites. To determine if Fos and Jun were present and might help mediate estrogen's effects on PR gene expression in MCF-7 cells, gel mobility shift assays were carried out with ^{32}P-labeled oligos

6

containing the higher affinity +745 AP-1 site and nuclear extracts from MCF-7 cells that had been treated with E_2 for 0, 2, 24 or 72 hours. Interestingly, a distinct protein-DNA complex was formed with nuclear extracts from MCF-7 cells that had been treated with E_2 for 72 hours (Fig. 5, Lane 5, ←), but not with extracts from cells that had been treated with E_2 for 0, 2, or 24 h (Lanes 2-4).

To determine if the protein-DNA complex formed with MCF-7 nuclear proteins contained Fos and/or Jun, gel mobility shift assays were carried out with ^{32}P-labeled oligos containing the +90 (Fig. 6, Panel A) or the +745 (Panel B) AP-1 site and nuclear extracts from MCF-7 cells that had been treated with E_2 for 72 h. Proteins present in the nuclear extracts bound to the +90 and +745 AP-1 sites (Lanes 2). However, significantly longer periods of exposure were required to detect the protein-DNA complex with the +90 AP-1 site. The protein-DNA complexes were disrupted by the Fos-specific antibody sc-52. This antibody recognizes c-Fos, but does not cross react with Fos B, Fra-1 or Fra-2 (Lanes 3). The protein-DNA complexes were also disrupted by the Jun-specific antibody sc-45. This antibody binds to c-Jun, but does not cross-react with Jun B or Jun D (Lanes 4). In contrast, the ER-specific antibody H151 (Lane 5) did not affect the protein-DNA complex. As seen in gel shifts with the purified Fos and Jun, more protein-DNA complex was formed when MCF-7 nuclear extracts were incubated with the +745 AP-1 site than with the +90 AP-1. These data indicate that Fos and Jun are present in MCF-7 nuclear extracts and that they bind to both the +90 and the +745 AP-1 sites. The abilities of both Fos and Jun antibodies to disrupt the protein-DNA complex suggests that the proteins bind to the AP-1 sites as a heterodimer.

Fos levels are increased and Jun is dephosphorylated after E_2 treatment of MCF-7 cells. Fos-Jun binding to the +90 and +745 AP-1 sites was enhanced after MCF-7 cells were treated with E_2 for

72 h. To understand how this increase in Fos-Jun binding might occur, we monitored the effects of E_2 on Fos and Jun in MCF-7 cells. Western blot analysis was performed with nuclear extracts from MCF-7 cells that had been treated with E_2 for 0, 2, 24, or 72 hours. The Fos-specific antibody identified two proteins with apparent molecular weights of 55 and 61 kD (Fig 7, Panel A, Fos ▶). The Jun-specific antibody identified proteins with apparent molecular weights of 39 and 42 kD (Jun ▶). Since both Fos and Jun can be phosphorylated (33-36), the presence of two bands for each of the proteins most likely represented different phosphorylation states of the AP-1 proteins.

Although Fos levels transiently increased after 2 hours of E_2 treatment, the levels of Jun did not change substantially after E_2 treatment. There was, however, an increase in the more rapidly migrating form of Jun and a concomitant decrease in the more slowly migrating form of Jun after 24 and 72 hours of E_2 treatment. To verify that Jun was dephosphorylated after exposure of MCF-7 cells to E_2, Western blot analysis was performed with nuclear extracts from MCF-7 cells that had been treated with E_2 for 0, 24, or 72 hours and the blot was probed with the Jun-specific antibody, sc-822, which recognizes only phosphorylated Jun. After 24 hours of E_2 treatment, there was a marked decrease in the level of phosphorylated Jun (Panel B). Phosphorylated Jun was nearly undetectable after 72 hours of E_2 treatment. Thus, both Fos and Jun are affected by E_2 treatment. Fos levels are transiently increased and Jun is dephosphorylated after E_2 treatment of MCF-7 cells.

ER is present at the +90 and +745 AP-1 sites in native chromatin. Our transient cotransfection assays documented that the +90 and +745 AP-1 sites could confer estrogen responsiveness to a heterologous promoter and suggested that the ER assists in conferring estrogen responsiveness to the PR gene. Previous studies have also suggested that ER may influence transcription through

direct interaction with Jun or indirectly through interaction with AP-1 bound coregulatory proteins (10, 11). To determine whether the ER was present at the +90 and +745 AP-1 sites in the endogenous PR gene residing in MCF-7 cells, chromatin immunoprecipitation (ChIP) assays were carried out. MCF-7 cells were treated with ethanol vehicle or E_2, exposed to formaldehyde to cross link proteins and DNA, and sonicated. ER-specific antibody or preimmune serum was used to immunoprecipitate protein-DNA complexes. The immunoprecipitated DNA was used as a template to generate discrete DNA fragments containing the +90 or +745 AP-1 site. Although genomic DNA that had not been subjected to immunoprecipitaion was readily amplified (C), amplified products were only obtained when the MCF-7 cells were treated with E_2 and an ER-specific antibody was used for immunoprecipitation (Fig. 8). No DNA fragments were obtained when cells were maintained in a hormone free environment ($-E_2$) or when preimmune serum (PI) was used for immunoprecipitation. To ensure that the ER-specific antibody precipitated only ER-containing protein-DNA complexes, we determined whether a region of the PR gene that contains neither an ERE nor an AP-1 site (-711 to -436) could be immunoprecipitated and amplified. Although neither preimmune serum nor ER-specific antibody was able to precipitate the -711 to -436 region of the PR gene, this region was readily amplified when genomic DNA was used as a control (C). These data demonstrate that the ER was associated with the Fos-Jun complex at the +90 and +745 AP-1 sites in the endogenous PR gene and that its association with these regions was strictly E_2 dependent.

DISCUSSION

The PR gene is induced by E_2 in normal mammary and uterine cells and in MCF-7 breast cancer cells (22-24). Although Promoters A and B impart E_2 responsiveness to the PR gene (28,

9

29), neither promoter contains an identifiable ERE. The 5' flanking region, however, does contain several putative transcription factor binding sites. The sequence and location of an ERE half site, a CAAT sequence, and Sp1 and AP-1 binding sites are conserved in mouse (37), rat (38), rabbit (39), and human (28) PR genes. This high degree of regulatory motif conservation suggests that multiple regions in the 5' flanking region are important in modulating PR gene expression. Thus, estrogen responsiveness of the PR gene may be imparted by the cooperative action of numerous regulatory sites rather than a single ERE.

Involvement of AP-1 sites in regulating PR gene expression

Genomic Southern analysis was used to identify an AP-1 site at +90 in the human PR gene that was hypersensitive to DNase I cleavage. This +90 AP-1 site and the +745 AP-1 site bound purified Fos and Jun, formed complexes with Fos-Jun heterodimers present in MCF-7 nuclear extracts, and functioned as E_2-responsive transcriptional enhancers in transient cotransfection assays. The region of the rabbit PR gene corresponding to the human +745 AP-1 site is identical in nucleotide sequence (Fig. 2). Interestingly, the AP-1 site in the rabbit gene overlaps with an imperfect ERE that has been implicated in regulating PR gene expression (32). Unlike the +745 AP-1 site, this ERE sequence is not conserved in the corresponding region of the human PR gene. A sequence identical to the +745 AP-1 site in the human gene is completely conserved in the mouse (37) and rat (38) PR gene. Thus, the location and nucleotide sequence of the +745 AP-1 site is identical in all four species. In contrast, the +90 AP-1 site is unique to the human PR gene.

The +90 (TGA_G_TGA) and +745 (TGA_C_TGA) AP-1 sites are nearly identical in nucleotide sequence. There were, however, distinct differences in the abilities of these two AP-1 sites to form protein-DNA complexes. More Fos-Jun-DNA complex was formed with the +745 AP-1 site than with the +90 AP-1 site in gel mobility shift assays. The enhanced ability of the

10

+745 AP-1 site to form a protein-DNA complex may be due to nucleotide sequence flanking the AP-1 sites. Nucleotide sequences flanking ovalbumin and collagenase AP-1 sites contribute to the abilities of these sites to mediate transcription activation (12).

Role of Fos and Jun in regulating PR gene expression

The AP-1 family of proteins is comprised of a number of polypeptides including Fos and Jun (35, 40). Fos and Jun heterodimerize to form the AP-1 complex that binds to AP-1 sites in target genes (41-43). Although Fos-Fos homodimers do not bind to the consensus AP-1 site, Jun-Jun homodimers can bind to and activate transcription through AP-1 sites (44). However, Jun-Jun homodimers are less potent in activating transcription than Fos-Jun heterodimers. Purified Fos and Jun, but neither Fos nor Jun alone, formed detectable complexes with the +90 and +745 AP-1 sites. Furthermore, Fos- and Jun-specific antibodies disrupted the protein-DNA complexes formed when MCF-7 nuclear extracts were combined with the +90 and +745 AP-1 sites. The fact that Fos-Jun heterodimers present in MCF-7 nuclear extracts interact with these two AP-1 sites *in vitro* suggests that they play a role in regulating expression of the human PR gene in MCF-7 cells *in vivo*.

Exposure of MCF-7 cells to E_2 caused a transient increase is Fos protein levels. This data is consistent with the transient increase in Fos mRNA levels that have been reported after a 1h treatment of MCF-7 cells to $E_{2(45)}$. These increases in Fos mRNA and protein levels may be mediated by interaction of the E_2-occupied receptor with an ERE in the 5' flanking region of the human fos gene (13) leading to increased gene expression.

Although the level of Jun was not substantially altered by E_2 treatment, there was a decrease in the level of phosphorylated Jun and a concomitant increase in the level of dephosphorylated Jun in MCF-7 cells with increasing times of E_2 treatment. Since

11

phosphorylation of Jun decreases AP-1 binding and activity (33, 46) and site-specific

dephosphorylation of Jun increases AP-1 binding activity (33), dephosphorylation of Jun may play

a role in mediating estrogen's effects on PR gene expression in MCF-7 cells. Estrogen-induced

phosphorylation of Jun has also been implicated in inhibiting differentiation of precursor cells to

osteoclasts through an AP-1 mediated pathway (47). Thus, by regulating the phosphorylation

state of Jun, estrogen-responsive gene expression may be activated or repressed. This could

require the combined efforts of a Jun-specific kinase and phosphatase. In fact, it has been

suggested that the latent, constituitively-phosphorylated Jun residing in unstimulated cells is

activated by a phosphatase to the active, dephosphorylated form (33, 48)

Estrogen-regulated expression of the PR gene

AP-1 sites play a critical role in mediating estrogen responsiveness of the ovalbumin, c-

fos, collagenase, and insulin-like growth factor genes (9, 12-14). We have demonstrated that

transcription of promoters containing the PR +90 or +745 AP-1 site was enhanced by E_2

treatment only in the presence of ER suggesting that both hormone and the receptor are required

for estrogen responsiveness of the PR gene. More importantly, E_2 treatment of MCF-7 cells,

which is required for activation of the PR gene, promoted the recruitment of ER to the +90 and

+745 AP-1 sites in the endogenous PR gene. The association of ER with the +90 and +745 AP-1

sites could be fostered by direct interaction of the ER amino terminus with Jun (10) and/or

simultaneous interaction of coactivators with the ER and AP-1 proteins (11). Our studies suggest

that estrogen's effects on PR gene expression are derived, in part, from modulation of Fos levels,

dephosphorylation of Jun, and the ability of the receptor itself to play a role in forming an active

transcription complex with Fos-Jun heterodimer at the +90 and +745 AP-1 sites. This is, to our

12

knowledge, the first demonstration that AP-1 sites residing in the human PR gene play a role in mediating estrogen-regulated gene expression.

The PR Promoters A and B contain a complex array of conserved transcription factor binding sites. We have provided evidence that two AP-1 sites help to confer estrogen responsiveness to the PR gene, but we believe that other sites are also involved in estrogen-regulated expression of the PR gene. In addition to influencing binding and activity of the +90 and +745 AP-1 sites in the PR gene, the ER enhances Sp1 binding to its recognition site and binds directly to an adjacent ERE half site in the PR Promoter A (15). Taken together, our combined studies suggest that the ER has direct and indirect effects on formation of an active transcription complex at the +90 and +745 AP-1 sites and that multiple transcription factors including Fos, Jun, ER, and Sp1 act in concert to confer estrogen responsiveness to the PR gene.

MATERIALS AND METHODS

Oligonucleotides and plasmid construction Oligos containing the +90 AP-1 site

(5'GATCTCACTTGTCATTTGAGTGAAATCTACAACCA3' and

5'GATCTGGTTGTAGATTTCACTCAAATGACAAGTGA3') and the +745 AP-1 site

(5'GATCTTCCAGTCGTCATGACTGAGCTGAAGGCAAA3' and

5'GATCTTTGCCTTCAGCTCAGTCATGACGACTGGAA3') were annealed and inserted into

Bgl II-cut, dephosphorylated chloramphenicol acetyl transferase (CAT) reporter plasmid, TATA-CAT (49), to create +90 or +745 AP-1 TATA-CAT, respectively containing two copies of each AP-1 site. The ligated vectors were transformed into the DH5α strain of *E. coli*, sequenced, and purified on two cesium chloride gradients.

13

<u>Cell culture</u> The osteosarcoma cell line U2-OS was maintained in Eagle's Minimum Essential Medium (MEM) with 10 µg/L phenol red and 15% heat-inactivated fetal calf serum. Media was changed to phenol red supplemented MEM with 5% charcoal dextran stripped (50) calf serum (Hyclone, Logan, Utah) four days prior to transfection and then transferred to phenol red free MEM supplemented with 5% charcoal dextran stripped calf serum two days prior to transfections. MCF-7 cells were maintained as previously described (15).

<u>Genomic Southern analysis</u> MCF-7 cells were exposed to ethanol vehicle or 10 nM E_2 for 24 hours, harvested, and washed twice with cold PBS. Pelleted cells were resuspended in hypotonic buffer (10 mM Tris, pH 7.5, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.2% NP-40, 0.15 mM spermine, 0.5 mM spermidine, 5% sucrose and 1 mM PMSF), homogenized, layered onto a sucrose cushion (10 mM Tris, pH 7.5, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 10 % sucrose and 1 mM PMSF), and centrifuged at 1600 x g for 10 min. The nuclear pellet was resuspended in wash buffer (10 mM Hepes, pH 7.5, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine and 0.5 mM spermidine) and centrifuged at 400 x g at 4°C. The pelleted nuclei were resuspended in wash buffer and treated with 0, 40, 80, 120, 160, 200, or 240 U DNase I / ml (Boehringer Mannheim, Indianapolis, IN) in DNase dilution buffer (10 mM Hepes, pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.1 mg/ml BSA) for 3 min at 4°C. Lysis solution (0.1 M EDTA, pH 8.0, 1% SDS, 0.4 mg proteinase K/ ml) was added to the mixture and incubated for 2 hours at 55°C. DNA was incubated with RNase A, phenol chloroform extracted, ethanol precipitated, resuspended in TE (10mM Tris pH 7.5, 1mM EDTA) and stored at -20°C. 10 µg of purified genomic DNA was digested with *Bam* HI (2 U/µg DNA) overnight at 37°C. The DNA was phenol chloroform extracted, ethanol precipitated, and resuspended in loading dye. The DNA was fractionated on a 1.5 % TBE agarose gel overnight at

20V. The gel was rinsed with distilled water, soaked in denaturing solution (0.5 M NaOH, 1.5 M

NaCl) at room temperature, rinsed with distilled water and soaked in neutralization buffer (1.5 M

NaCl, 0.5 M Tris, pH 7.2, 0.001 M EDTA) at room temperature for 30 min. The DNA was

transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA) in 20X SSC, and UV cross-

linked. The membrane was prehybridized with 10 ml of Rapid-hyb buffer (Amersham Pharmacia,

Piscataway, NJ) for 2 hours at 65°C. A 300 basepair polymerase chain reaction (PCR) product

from the human PR gene (-714 to -436) was produced using the pBL3 B promoter (kindly

provided by Pierre Chambon, Strasbourg, France), ^{32}P-labeled using random primers, and

incubated with the membrane overnight at 65°C. The membrane was washed twice with 2X SSC

and 0.5% SDS and once with 0.2X SSC and 0.5% SDS at room temperature for 15 min.

Radioactive bands were visualized by autoradiography.

Transient cotransfections 4 x 10^5 U2-OS cells were plated in each well of a 24 well plate the day

before transfection. Transfections were carried out using lipofectin (Gibco BRL, Grand Island,

NY) as described previously (51) with 7.5 µg of the indicated reporter vector and 150 µg of the

β-galactosidase vector CMVβ-gal (Promega, Madison WI). 100 ng of the human ERα expression

vector CMV5hER (52) was added as indicated. Cells were maintained in media containing

ethanol vehicle or 10 nM E$_2$ for 24 hours. β-galactosidase activity was determined at room

temperature as previously described (53) and used to normalize the amount of CAT activity in

each sample. CAT assays were carried out as described (51).

Gel mobility shift assays Gel mobility shift assays were carried out essentially as described (15,

54). ^{32}P-labeled (10,000 cpm) oligos containing the +90 or +745 AP-1 site were combined with

either 20 µg nuclear extract from MCF-7 cells that had been treated with E$_2$ for 0, 2, 24 or 72

hours or 0.005-0.07 µM purified Fos and Jun protein (kindly provide by Tom Kerppola,

University of Michigan School of Medicine, Ann Arbor, MI) in binding buffer (15 mM Tris, pH 7.9, 0.2 mM EDTA, 10% glycerol, 50 mM KCl, 1 mM $MgCl_2$, 50ng of poly dI/dC and 0.4 mM DTT) for 15 min at room temperature in a final volume of 20 µl. BSA, ovalbumin and KCl were included as needed to maintain constant protein and salt concentrations. When MCF-7 nuclear extracts were used, the nonspecific DNA for each reaction included 1µg of salmon sperm DNA and 2 µg of poly dI/dC. BSA was used with purified proteins so that the total protein concentration in each reaction was 20 µg. For antibody supershift experiments, the Fos-specific antibody, sc-52, (Santa Cruz Biotech, Santa Cruz, CA), Jun-specific antibody, sc-45 (Santa Cruz Biotech, Santa Cruz, CA) or ER-specific monoclonal antibody H151 (kindly provided by Dean Edwards, University of Colorado Health Science Center, Denver, CO) was added to the protein-DNA mixture and incubated for 10 min at room temperature. Low ionic strength gels and buffers were prepared as described (55). Radioactive bands were visualized by autoradiography.

Preparation of MCF-7 nuclear extracts and purified Fos and Jun MCF-7 cells were exposed to 10 nM E_2 for 0, 2, 24, or 72 hrs, harvested, and pelleted. Pelleted cells were resuspended in 400 ul of TEG (50 mM Tris pH 8.5, 7.5 mM EDTA, 10% glycerol), homogenized and centrifuged at 10,200 x g for 10 sec. The nuclei were resuspended in TEG containing 0.5mM KCl and incubated at 4°C for 20 min. The nuclear extract was centrifuged at 150,000 x g for 30 min, aliquoted, assayed for protein concentration, and stored at -80°C. The expression and purification of Fos and Jun have been described (56-59).

Western blots 20 µg of MCF-7 nuclear extract was fractionated on a 10% SDS acrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with $T_{50}BS$ solution (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM thimerosal) containing 5% Carnation nonfat dry milk for 1 hour at room temperature. The Fos antibody, sc-52, was diluted 1:2000 in a 5% milk

16

$T_{50}BS$ solution. The Jun antibodies, sc-45 and sc-822, were diluted 1:1000 or 1:200,

respectively, in a 5% milk $T_{50}BS$ solution. Antibodies were incubated with the membrane for 1

hour at room temperature. Blots were washed with $T_{50}BS$ with 0.05% Tween 20 and incubated

with a horse radish peroxidase coupled secondary goat anti-rabbit IgG, sc-2004, or rabbit anti-

mouse IgG, sc2005, (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50,000 and 1:10,000

dilution, respectively, in 5% milk $T_{50}BS$ solution. Blots were washed, incubated with Supersignal

Luminescent Substrate (Pierce, Rockford, IL), and exposed to film.

Chromatin immunoprecipitation assays. ChIP assays were carried out essentially as described in

Upstate Biotechnology Tech Note 50105 (Upstate Biotechnology, Waltham, MA). The ER-

specific antibody sc-8002 (Santa Cruz Biotech, Santa Cruz, CA) or mouse preimmune serum was

used for immunoprecipitation of protein-DNA complexes. PCR primers flanking the +90 (5'-

GGCTTTGGGCGGGGCCTCCCTA-3' and 5'-TCTGCTGGCTCCGTACTGCGG-3') or +745

(5'-TTCTCCTCCCTCTGCCCCTATATTCCCG-3' and 5'-GGCGACACAGCAGTGGGGAT-

3') AP-1 sites produced 234 or 188 basepair DNA fragments, respectively. As a negative control,

primers that annealed from -711 to -693 and from -458 to -436 of the PR gene were used to

produce a 275 bp fragment. This region of the PR gene does not contain an identifiable ERE, Sp1

or AP-1 site.

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FIGURE LEGENDS

Figure 1. Genomic Southern analysis of the human PR gene MCF-7 cells were maintained in serum-free medium for five days and then treated with ethanol control (-E_2, lanes 1-7) or 10 nM E_2 (+E2, lanes 8-14) for 24 hours. Nuclei were isolated and incubated with increasing concentrations of DNase I. Genomic DNA was purified, fractionated on a nondenaturing gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled fragment that annealed to the -714 to -436 region of the PR gene. Gels were visualized by autoradiography. The locations of hypersensitive bands are indicated ($\leftarrow$).

Figure 2. Sequence of human progesterone receptor AP-1 sites The sequence of the +745 AP-1 site in the human PR gene is shown in bold. The corresponding sequences of the rabbit, mouse, and rat PR genes are also included. The boxed area represents the translation start site for PR-B.

Figure 3. Transient cotransfections with reporter plasmids containing AP-1 sites U2-OS cells were transfected with a reporter plasmid containing two copies of the +90 (+90 AP-1 TATA-CAT) or +745 (+745 AP-1 TATA-CAT) AP-1 site or a TATA box alone (TATA-CAT) and a β-galactosidase expression plasmid. A human ER expression plasmid was (+ER) or was not (-ER) included as indicated. Cells were treated with ethanol vehicle or 10 nM E_2 and CAT activity was determined. Data from 6 independent experiments were combined and values are presented as the mean ± SEM. Student's t tests demonstrated that the E_2-treated samples were statistically different from the corresponding ethanol-treated samples when the ER expression plasmid was included and reporter plasmids contained the +90 or +745 AP-1 site ($p<10^{-6}$).

27

Figure 4. Gel mobility shift assays with AP-1-containing oligos and purified Fos and Jun ^{32}P-labeled oligos containing the +90 (Panel A) or the +745 (Panel B) AP-1 sites were incubated alone (Lane 1) or with 0.005, 0.01, 0.02, 0.05 (Lanes 2-5) or 0.07 μM (Lanes 6-8) purified Fos and Jun. Fos-(α Fos) or Jun-(α Jun) specific antibody was added to the binding reaction as indicated.

Figure 5. Gel mobility shift assays with AP-1-containing oligos and MCF-7 nuclear proteins ^{32}P-labeled oligos containing the +745 AP-1 site were incubated alone (Lane 1) or with 20 μg of nuclear extracts from 0, 2, 24 or 72 hour E_2-treated MCF-7 cells (Lanes 2-5). The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

Figure 6. Antibody supershift assays with AP-1-containing oligos and MCF-7 nuclear proteins ^{32}P-labeled oligos containing the +90 (Panel A) or +745 (Panel B) AP-1 site were incubated alone (Lane 1) or with 20 μg of nuclear extracts from MCF-7 cells that had been treated with E_2 for 72 hours (Lanes 2-5). Fos-(Lane 3, α Fos), Jun-(Lane 4, α Fos), or ER-specific antibody (Lane 5, α ER) was added to the binding reaction as indicated. The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

Figure 7. Western Blot analysis of nuclear extracts from E_2-treated MCF-7 cells 20 μg of nuclear extract from 0, 2, 24 or 72 hours of E_2-treated MCF-7 cells were fractionated on a denaturing gel, transferred to a nitrocellulose membrane and incubated with a Fos-specific antibody, an antibody that recognizes phosphorylated and dephosphorylated Jun (Panel A), or an antibody that

recognizes phosphoylated, but not dephosphorylated, Jun (Panel B). A chemiluminescent substrate was used to detect the bands.

Figure 8. Chromatin immunoprecipitation of ER-associated DNA. MCF-7 cells, which had been exposed to ethanol vehicle or 10 nM E_2, were treated with 1% formaldehyde to cross link protein and DNA and then sonicated. Protein-DNA complexes were immunoprecipitated with preimmune serum (PI) or ER-specific (αER) antibody. After crosslink reversal and DNA purification, primers flanking the +90 or +745 AP-1 site or a region that did not contain an AP-1 site (-711/-436) were used in PCR amplification. Amplified DNA was fractionated on an agarose gel and visualized after ethidium bromide staining. Genomic DNA was used as a positive control (C).
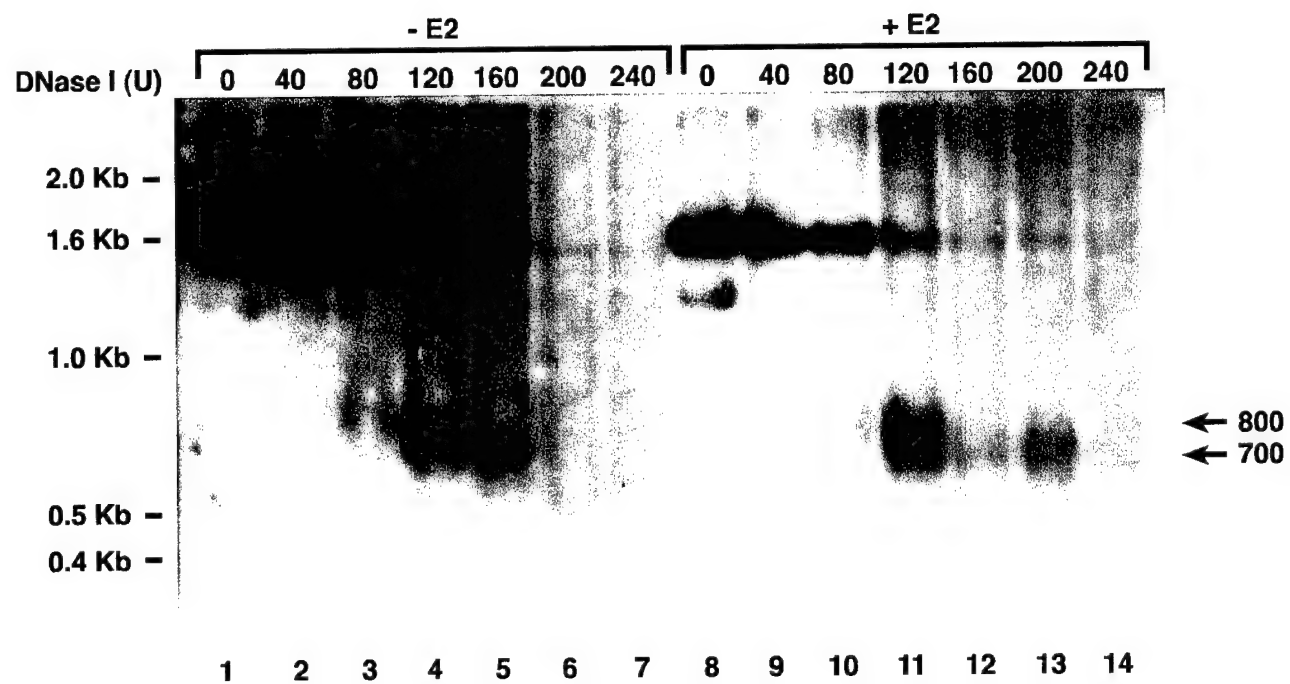
Figure 1

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Human  +735 TCCAGTCGTCATGACTGAGCTGA +756
Rabbit +703 TCAGGTCGACATGACTGAGCTGA +725
Mouse  +625 TTGGGTCGTCATGACTGAGCTGC +647
Rat    +617 TCGGGTCGTCATGACTGAGCTGC +639
```
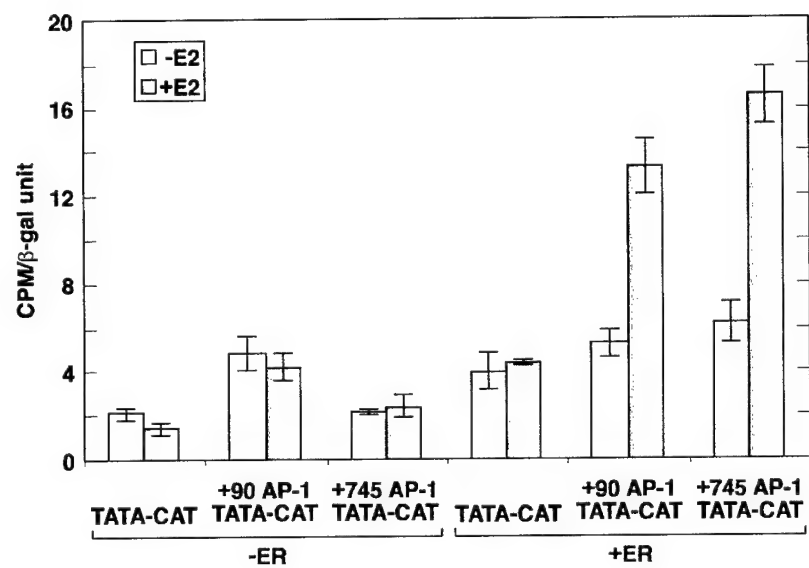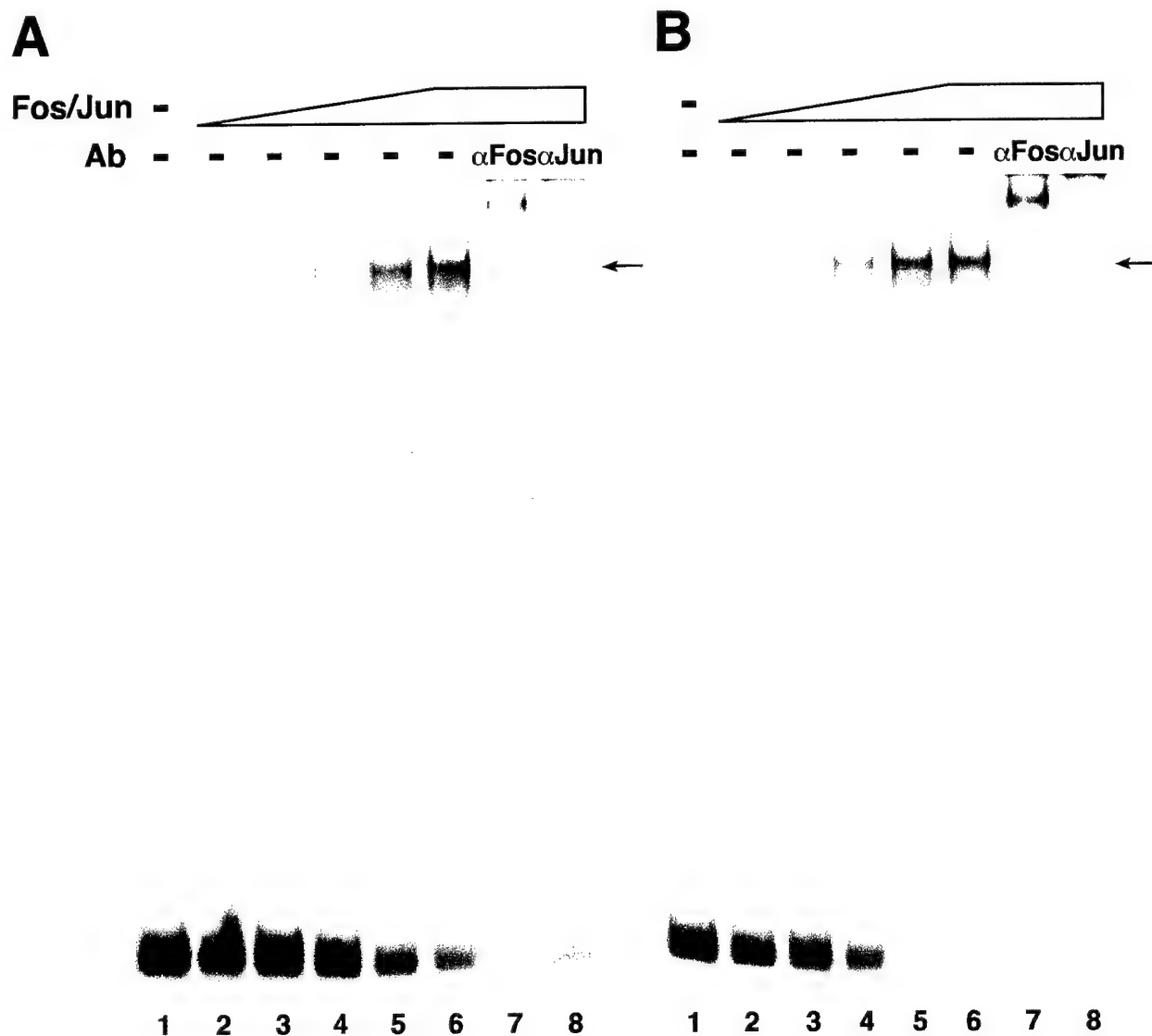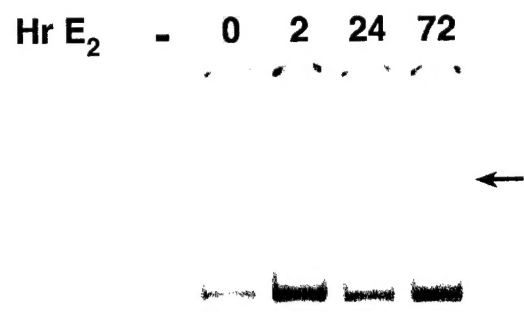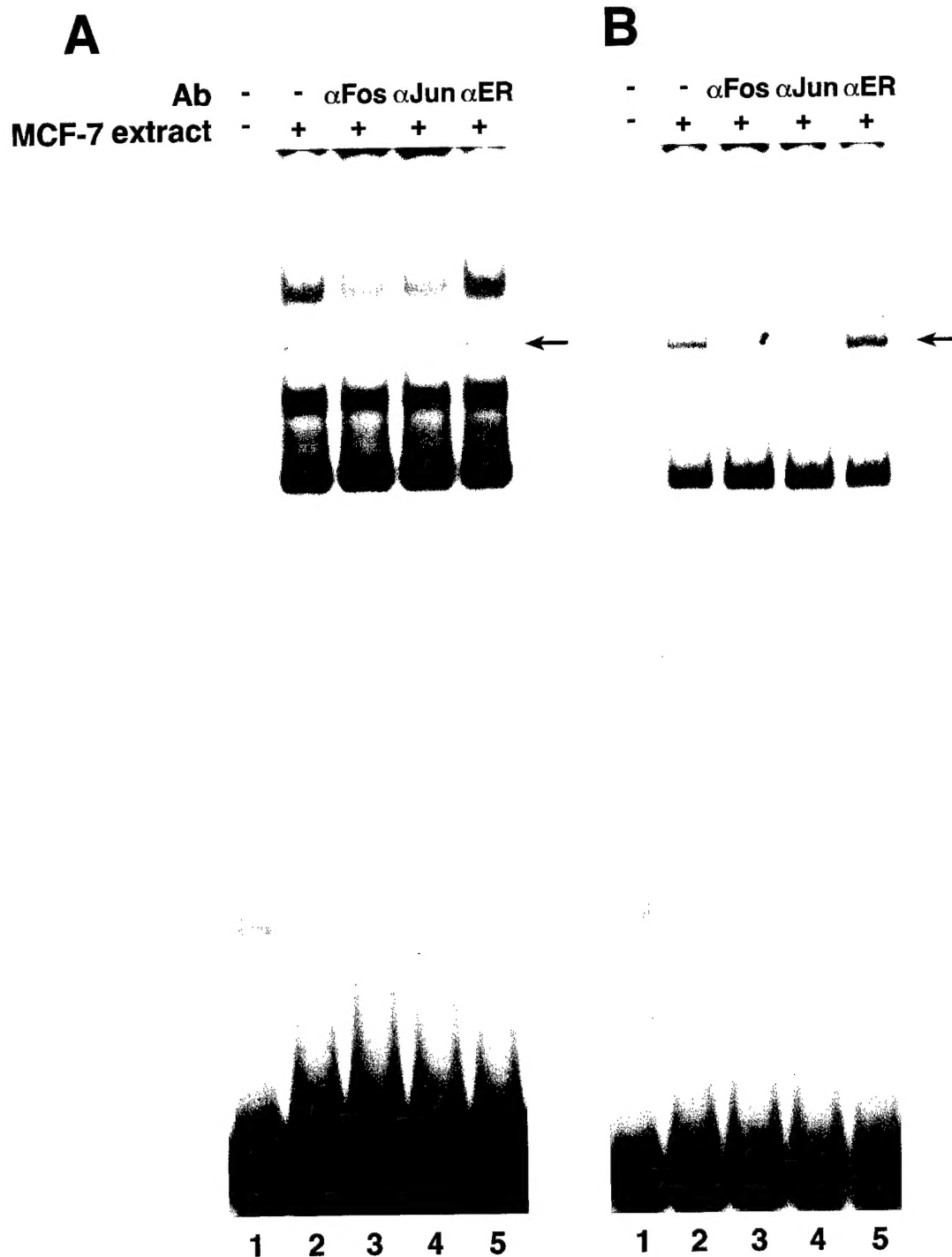
Figure 2

Figure 3

A

Fos/Jun -

Ab   -   -   -   -   -   -   αFosαJun

B

-

-    -   -   -   -   -   αFosαJun

1   2   3   4   5   6   7   8   1   2   3   4   5   6   7   8

Figure 4

Hr E$_2$ -   0   2   24   72

←

1   2   3   4   5

Figure 5

A

| Ab | - | - | αFos | αJun | αER |
| MCF-7 extract | - | + | + | + | + |

←

B

| | - | - | αFos | αJun | αER |
| | - | + | + | + | + |

←

1 2 3 4 5   1 2 3 4 5

Figure 6

A

Hr E_2

| 0 | 2 | 24 | 72 |

Fos⟩

Jun⟩

B

Hr E_2

| 0 | 24 | 72 |

Jun⟩

Figure 7

Figure 8